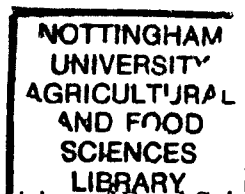


Ferulic Acid Esterases for Effective Processing of Plant Carbohydrates

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ABSTRACT

Feruloyl esterases (E.C. 3.1.1.73), a subclass of the carboxylic acid esterases (E.C. 3.1.1.1), are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in the plant cell walls, and have been classified as Types A or B based on their substrate specificity for aromatic moieties. They constitute an interesting group of enzymes that have the potential for use over a broad range of applications in the agri-food industries. In order to expand the range of available enzymes, we have examined the production of feruloyl esterases by the filamentous fungi *Talaromyces stipitatus* and *Neurospora crassa*.

Neurospora crassa has been shown to produce multiple feruloyl esterase activities depending upon the time of fermentation with either sugar beet pulp or wheat bran substrates. A gene identified on the basis of its expression on sugar beet pulp has been cloned and over-expressed in *Pichia pastoris*. The gene encodes a single domain feruloyl esterase (NcFae-1), which represents the first report of a non-modular Type-B enzyme and the purified recombinant protein has been shown to exhibit concentration dependent substrate inhibition. The kinetic behaviour of the non-modular enzyme is discussed in terms of the diversity in the roles of the feruloyl esterases in the mobilisation of plant cell wall materials and their respective modes of action.

A novel feruloyl esterase (TsFAEC, Type-C feruloyl esterase) that exhibits broad substrate specificity in culture supernatants of *Talaromyces stipitatus* when grown on sugar beet pulp, has been cloned and over-expressed in *Pichia pastoris*. Various gene fusions have been constructed to investigate the use of alternative signal peptides by *P. pastoris* and to produce an authentic feruloyl esterase featuring the N-terminal sequence determined for the native enzyme. It has been demonstrated that additional amino acids at the N-terminus of the FAEC sequence do not influence the catalytic capacity of the enzyme, and that the nature of the signal sequence does not appreciably alter the yield of the secreted enzyme.

NcFae-1 and TsFAEC contain internal peptide sequences that correspond with the consensus motif G-X-S-X-G that contains the catalytic serine nucleophile conserved in the esterase enzyme superfamily. The serine residues at the centre of these peptide motifs have been independently mutated and the corresponding enzymes over-expressed in *P. pastoris* to identify essential serine residues as candidate nucleophiles responsible for catalysing the enzymatic reaction.

Based on activity profile data and supported by the characterisation of a recombinant Type-D feruloyl esterase from *N. crassa*, a feruloyl esterase sub-classification is proposed and discussed in terms of the evolutionary relationships existing between carbohydrate esterases.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	Alanine (one-letter code)
<i>A. oryzae</i>	<i>Aspergillus oryzae</i>
ACE	Acetyl esterase
Ala	Alanine (three-letter code)
AMV	<i>Avian myeloblastosis Virus</i>
AnFAEA	FAEA feruloyl esterase from <i>Aspergillus niger</i>
AnFAEB	FAEB feruloyl esterase from <i>Aspergillus niger</i>
<i>A. niger</i>	<i>Aspergillus niger</i>
<i>AOXI</i>	<i>Pichia pastoris</i> alcohol oxidase I gene
APS	Ammonium persulphate
Ara ₂ F	[2- <i>O</i> -(<i>trans</i> -feruloyl)- α -L-arabinofuranosyl]-(1,5)-L-arabinofuranose
Asp	Aspartic acid (three-letter code)
ATP	Adenosine triphosphate
<i>Av</i> II	restriction enzymes cutting at TGC~GCA
AXE	Acetylxyln esterase
<i>Bam</i> HI	restriction enzymes cutting at G~GATCC
BCIP	5-bromo-4-chloro-3-indolyl phosphate
<i>Bln</i> I	restriction enzymes cutting at C~CTAGG
bp	base pair
BSA	Bovine serum albumin
BSG	Brewer's spent grain
C	Cysteine (one-letter code)
°C	degrees Celsius
CBD	Cellulose Binding Domain
cDNA	complementary DNA
cfu	Colony-forming unit
χ^2	Chi square = $\sum \{ (O-E)^2 / E \}$; with O = Observed frequency and E = Expected frequency.
CIAP	Calf intestinal alkaline phosphatase
CIP	Calf intestinal phosphatase

cm	Centimetre
C-terminus	Carboxyl terminus of a protein
D	Aspartic acid (one-letter code)
Da	Dalton
DiFA	Diferulate
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNS	Dinitrosalicylic acid
dNTP	2' deoxyribonucleotide 5' triphosphate
<i>DraI</i>	restriction enzymes cutting at TTT [*] AAA
DTT	Dithiothreitol
E.C.	Enzyme Classification
<i>E. coli</i>	<i>Escherichia coli</i>
<i>EcoRI</i>	restriction enzymes cutting at G [*] AATTC
EDTA	Ethylene diamine tetra-acetic acid
FAE	Feruloyl esterase, ferulic acid esterase, cinnamoyl esterase, cinnamic acid hydrolase (EC 3.1.1.72)
FAXX	[5- <i>O</i> —(<i>trans</i> -feruloyl)- α -L-arabinofuranosyl]-(1,3)- β -D-xylopyranosyl-(1,4) D-xylopyranose
G	Glycine (one-letter code)
Gly	Glycine (three-letter code)
His	Histidine (three-letter code)
His ⁺	phenotype of <i>Pichia pastoris</i> referring to histidinol dehydrogenase activity
<i>his4</i>	<i>Pichia pastoris</i> histidinol dehydrogenase gene
HPLC	High pressure liquid chromatography
Ile	Isoleucine (three-letter code)
IPTG	Isopropyl- β -D-thiogalactoside
kb	kilobase pair
k_{cat}	Catalytic constant (or turnover number) is the number of substrate molecules converted to product per enzyme molecule per unit of time (seconds), when the enzyme is saturated with substrate.

k_{cat} / K_m	Catalytic efficiency
K_i	Inhibition constant or dissociation constant of the ESS complex ($K_i = ([\text{ES}] [\text{S}]) / [\text{ESS}]$; with E = enzyme and S = substrate)
K_m	Michaelis constant (K_m = substrate concentration corresponding at $V_{\text{max}} / 2$)
<i>Kpn</i> I	restriction enzymes cutting at GGTAC~C
<i>lacZ</i>	β -galactosidase gene
LB	Luria-Bertani medium
Leu	Leucine (three-letter code)
M	Molar
mA	milliamperes
MCA	Methyl caffeate (methyl 3,4-dihydroxycinnamate)
MCS	Multiple cloning site
MD	Minimal dextrose
Met	Methionine (three-letter code)
μF	Microfarad (unit of capacitance)
MFA	Methyl ferulate (methyl 3-methoxy-4-hydroxycinnamate)
ml	millilitre
MM	Minimal methanol
mol	mole
MOPS	3-(N-Morpholino)-propane sulfonic acid
<i>Mp</i> CA	Methyl <i>p</i> -coumarate (methyl 4-hydroxycinnamate)
mRNA	messenger RNA
MSA	Methyl sinapate (methyl 3,5-dimethoxy-4-hydroxycinnamate)
Mut ^{+/S}	Methanol utilisation phenotype of <i>Pichia pastoris</i> GS115
NBT	Nitroblue tetrazolium
NcFae-1	Fae-1 feruloyl esterase from <i>Neurospora crassa</i>
<i>N. crassa</i>	<i>Neurospora crassa</i>
<i>Nde</i> I	restriction enzymes cutting at CA~TATG
<i>Not</i> I	restriction enzymes cutting at GC~GGCCGC
<i>Nsi</i> I	restriction enzymes cutting at ATGCA~T
N-terminus	Amino terminus of a protein
OD	Optical density

PAGE	Polyacrylamide gel electrophoresis
P _{AOXI}	<i>AOXI</i> promoter
PCR	Polymerase chain reaction
PfFAEB	FAEB feruloyl esterase from <i>Penicillium funiculosum</i>
<i>P. fluorescens</i>	<i>Pseudomonas fluorescens</i>
pfu	Plaque-forming unit
<i>P. funiculosum</i>	<i>Penicillium funiculosum</i>
PfXYLD	XYLD acetyl esterase from <i>Pseudomonas fluorescens</i> subsp. <i>cellulosa</i>
Phe	Phenylalanine (three-letter code)
<i>P. pastoris</i>	<i>Pichia pastoris</i>
Pro	Proline (three-letter code)
PVDF	Polyvinylidene difluoride
RACE	Rapid amplification of cDNA ends
RLM	RNA ligase-mediated
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
rRNA	Ribosomal RNA
RT	reverse transcriptase
S	Serine (one-letter code)
<i>Sau3AI</i>	restriction enzymes cutting at 5'-GATC
Ser	Serine (three-letter code)
SBP	Sugar beet pulp
SD	Sabouraud-dextrose medium
SDS	Sodium dodecyl sulphate
<i>SnaBI</i>	restriction enzymes cutting at TAC 5'-GTA
TAE	Tris-acetate EDTA buffer
<i>Tal. stipitatus</i>	<i>Talaromyces stipitatus</i>
TAP	Tobacco acid pyrophosphatase
TEMED	N,N,N',N'-tetramethyl ethylene diamine
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophane (three-letter code)

TsFAEC	<i>Talaromyces stipitatus</i> FAEC
<i>T. viride</i>	<i>Trichoderma viride</i>
U	Unit
UV	Ultraviolet
V	Volt
V _{max}	maximum velocity
v/v	volume for volume
Ω	Ohm (unit of resistance)
WB	Wheat bran
WIAX	Water insoluble arabinoxylans
WSAX	Water soluble arabinoxylans
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-β-D-galactopyranoside
<i>Xho</i> I	restriction enzymes cutting at C [*] TCGAG

PREFACE

This PhD thesis is an outcome of a project funded by the Biotechnology and Biological Sciences Research Council – UK (BBSRC) and the Department of Trade and Industry – UK (DTI) through the LINK APPLIED BIOCATALYST programme (Grant no. BTL/27/12). The project entitled “Feruloyl Esterases for Effective Processing of Plant Carbohydrates”, involves collaboration between the University of Nottingham, the Institute of Food Research, Norwich, and the companies Biocatalyst Ltd. and Archers Daniel Midland (ADM).

CHAPTER 1. INTRODUCTION

This PhD thesis is an outcome of an Applied Biocatalyst LINK project entitled “Ferulic Acid Esterases for Effective Processing of Plant Carbohydrates”.

Feruloyl esterases have a key role to play amongst the battery of enzymes required to digest plant cell walls. This thesis is a report of my contribution to a wider LINK project instigated to characterise feruloyl esterases and assess their role together with synergistic activities as useful biocatalysts in plant cell-wall modification. These feruloyl esterase/carbohydrase combinations will be utilised to extract ferulic acid from agro-industrial waste materials in order to use these natural materials for food, health and cosmetic use.

Sequential extraction of plant cell wall polysaccharides can theoretically be achieved either chemically or enzymatically. While chemical extractions can solubilise large polysaccharides, carbohydrate substituents such as acetyl, methyl, and feruloyl ester-linked groups can be lost. The use of harsh chemicals, such as alkalis and acids, may also modify the polymeric sugars or partially degrade them in oxidative reactions. The use of highly specific enzymes, on the other hand, may be used to remove polymeric structures attached to a polysaccharide backbone of interest, facilitating the solubilisation of this material, and retaining much of their intricate detailed structure. Enzymes can also be used to release specific components of the plant cell wall such as phenolic derivatives for use in food industry. However, for effective processing of plant carbohydrates, one enzyme working alone is often not sufficient. Functional combinations of enzymes must be identified to release the products of interest.

The work contained in this thesis reports the molecular cloning of novel feruloyl esterase genes, their over-expression in heterologous expression systems and the functional characterisation of the recombinant enzymes.

1.1. PHENOLIC RESIDUES IN PLANT CELL WALLS

1.1.1. The plant cell wall

The cell wall is a layer of structural material found external to the protoplast. It is usually 0.1 - 10 μm thick and composed of polysaccharides, with smaller quantities of glycoproteins and phenolic compounds. Some of these structures are among the most complex known. The polysaccharide framework gives the cell wall its basic shape, size and thickness. Primary and secondary structural cell walls differ in their chemical composition. This is not surprising regarding the different biological roles of the two structures. The primary wall has to be resistant but plastic enough to allow growth. Its polysaccharide framework is deposited during growth at the cell surface. Secondary wall material, however, is deposited after the cessation of surface growth. Its biological roles are defence, support and storage. Lignin accumulated in the secondary walls will prevent further growth. The polysaccharides of the primary cell wall are classified as pectic, hemicellulosic and cellulosic. Table 1-1 summarizes the polymer composition of the primary walls of grasses (Monocot) and higher plants (Dicots).

Table 1-1: Polymer composition of the primary walls of grasses (Monocot) and higher plants (Dicots) (Fry, 1988). ND: not detected.

Polymer		% of dry weight of unligified primary cell wall	
		Monocot	Dicot
Cellulose	polysaccharides	30	30
Pectin		5	35
Arabinoxylan		30	5
β -(1-3), (1-4)-glucan		30	ND
Xyloglucan		4	25
Extensine	glycoprotein	0.5	5

Cellulose is an unbranched polymer of D-glucopyranose residues joined by β -(1-4) linkages. Cellulose constitutes between 20 - 30 % of the dry weight of the primary wall and 40 - 90 % of the secondary wall of most plants, and as such is the world's most abundant organic compound.

Pectins are complex molecules comprising of “smooth” and “hairy” regions. The smooth regions (or homogalacturonans) consist mainly of contiguous unbranched α -(1-4) linked galacturonic acid (pyranose) residues. The “smooth” regions are interspersed with “hairy” regions composed of rhamnogalacturonans bearing arabinan and/or (arabino)galactan side-chains linked to rhamnose residues (Figure 1-1). The primary cell walls of dicotydous plants are rich in pectins, whereas only traces of pectins are found in grasses (Saulnier and Thibault, 1999; Williamson *et al.*, 1998b).

Hemicelluloses are non-cellulosic wall polysaccharides other than pectins. The principal hemicelluloses are xylans. Xylans constitute 5 % of the primary cell wall in Dicots but 20 % of the walls of grasses, and 20 % of the secondary walls of both Dicots and Monocots. Xyloglucans, however, constitute 20 % of the primary cell wall of Dicots, and 1 - 5 % of the primary walls of grasses. Finally, β -(1-3), (1-4)-glucans, are abundant polysaccharides of the cell walls of grasses (Figure 1-2) but are not a major compound in Dicots (Figure 1-1).

Certain primary walls contain lignin in addition to the polymers listed above. Lignin is a phenolic polymer formed from *p*-coumaryl-, coniferyl- and sinapyl-alcohols. It is the second most abundant organic substance on Earth, making up 20 - 30 % of the dry weight of wood. Lignin is a hydrophobic filler, replacing the wall's water and permanently preventing further growth. Growing walls contain little or no lignin but traces of phenolics that are present may nevertheless act as important linking sites between polysaccharides.

Figure 1-1 and Figure 1-2 illustrate how the polymer composition of the primary walls of higher plants (Dicots) differs considerably from grasses (Monocots).

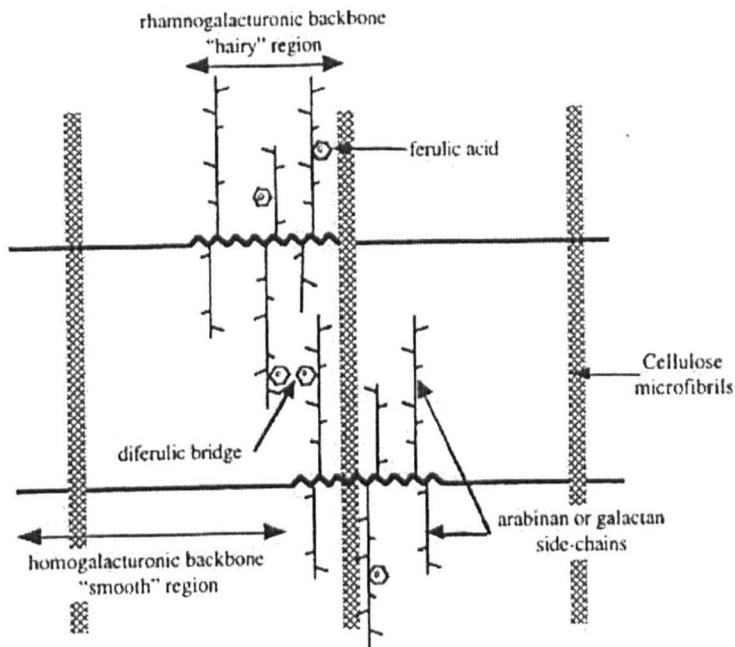


Figure 1-1: Model of Sugar beet (Dicots) cell wall (Saulnier and Thibault, 1999).

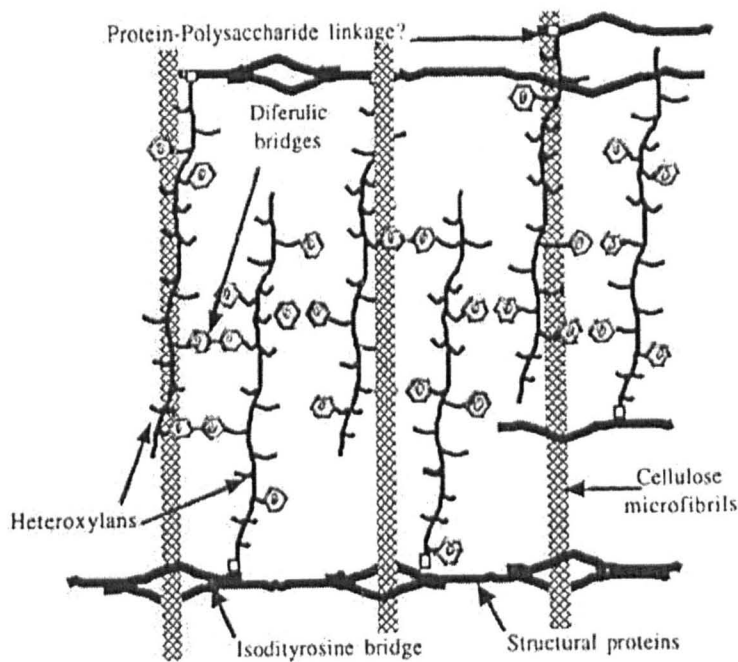


Figure 1-2: Model of grasses (Monocots) cell wall (Saulnier and Thibault, 1999).

1.1.2. Wall phenolics

1.1.2.1. Hydroxycinnamic acids

1.1.2.1.1. Phenolics in plant cell walls

A phenolic is a compound with an –OH group attached directly to a benzene ring; the simplest is phenol itself, C_6H_5OH . Cell wall hydroxycinnamates are derived from the phenylpropanoid pathway, which originates from phenylalanine and tyrosine (Brett *et al.*, 1999). Hydroxycinnamic acids, such as ferulic (FA: 3-methoxy-4-hydroxycinnamic), sinapic (SA: 3,5-dimethoxy-4-hydroxycinnamic), caffeic (CA: 3,4-dihydroxycinnamic) and *p*-coumaric acid (*p*CA: 4-hydroxycinnamic) are found both covalently attached to the plant cell wall and as soluble forms in the cytoplasm (Figure 1-3). There is evidence that ferulic acid is esterified to wall polymers intracellularly from feruloyl-CoA, which itself derived from the amino acids phenylalanine and tyrosine (Brett *et al.*, 1999; Meyer *et al.*, 1991).

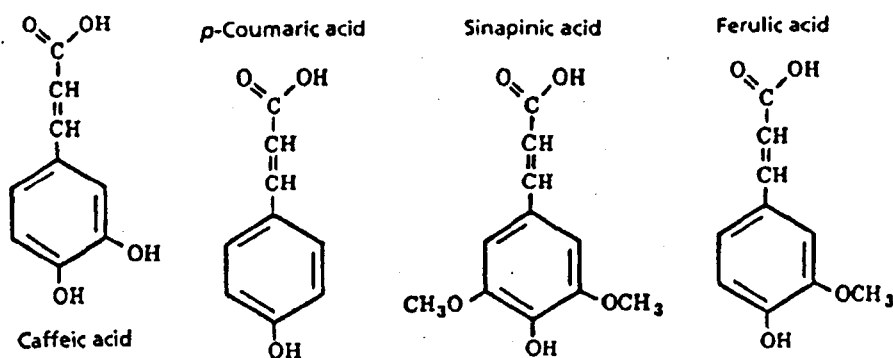


Figure 1-3: Structure of common cell wall hydroxycinnamic acids (Faulds and Williamson, 1993).

Aromatic compounds in the plant cell wall are almost exclusively derived from ferulic, *p*-coumaric, caffeic and sinapic acids (Figure 1-3). In the plant cell wall, ferulic and *p*-coumaric acids are bound through an ester linkage to the arabinoxylans

of gramineae or to the pectins of dicotyledons. Ferulic acid is the most abundant member of this class of phytochemicals. It is distributed throughout the primary and secondary cell walls of plants (Clifford, 2000; Fry, 1982). While *p*-coumaric acid tends to be the main hydroxycinnamate in the stems/stalks of cereals (Faulds and Williamson, 1999a), higher proportions of ferulic acid are covalently bound through an ester link to xylans, pectins and xyloglucans in the cell walls of species such as cereals (Harris and Hartley, 1977; Ralph *et al.*, 1992), sugar beet (Rombouts and Thibault, 1986), spinach (Fry, 1982), bamboo (Ishii *et al.*, 1990) and Chinese water chestnut (Parr *et al.*, 1996), and constitute part of the polyaromatic domain of suberin (Bernards *et al.*, 1995).

Table 1-2: Level of hydroxycinnamates in common fruits and vegetables (Clifford, 2000).

Hydroxycinnamate	Main dietary source	Levels (mg/kg)
Ferulic acid	Coffee, citrus juice, cereal brans, sugar beet fibre	700-3 000
Caffeic acid	Coffee, apples, cider, blueberries	12 500-37 500
Sinapic acid	Broccoli, kale, citrus juice, rice	>100
<i>p</i> -Coumaric acid	Spinach, cereal brans, sugar beet fibre, citrus fruits	45-350

The plant cell wall of graminaceous monocots such as wheat, maize, barley, contain up to 12 % of their dry weight as phenolic material and up to 3 % (w/w) ferulic acid which is ester-linked at O-5 of arabinofuranose substituents of the arabinoxylans (Figure 1-4). A few dicots, including sugar beet and spinach, contain up to 1 % (w/w) ferulic acid, which is esterified either at O-2 of arabinopyranose (50 - 55 % of the feruloyl groups) or at O-6 of galactopyranose residues in the pectic side chains

(45 - 40 % of the feruloyl groups) (Kroon *et al.*, 1999; Ralet *et al.*, 1994c), (Figure 1-4).

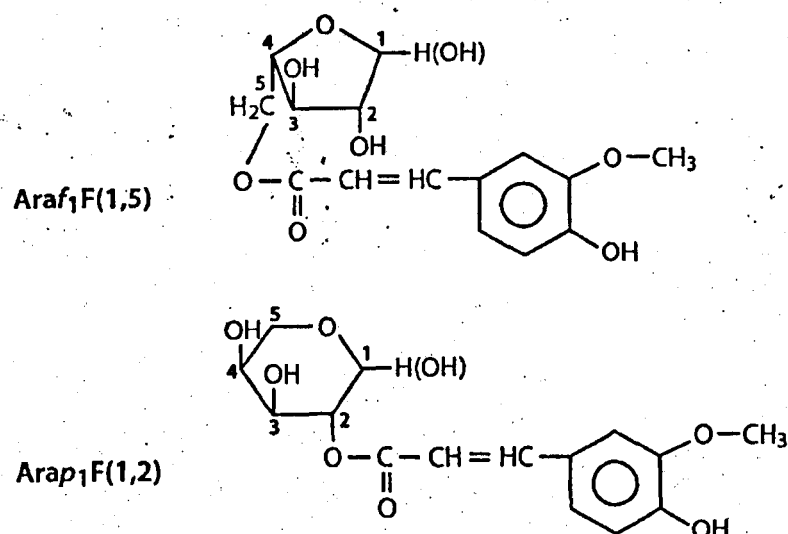


Figure 1-4: Ester linkages between arabinosyl residues and ferulic acid (Williamson *et al.*, 1998a).

1.1.2.1.2. Phenolic properties

1.1.2.1.2.1. Charge

Phenolic hydroxyl groups are negatively charged at high pH ($pK_a = 8 - 10$). The pK_a however is lower in feruloyl esters (8.6) than in ferulic acid (9.2). In some phenolics such as the dityrosines, the pK_a of the first hydroxyl ionisation can be low (6.7) when compared to the pK_a of second hydroxyl group (Fry, 1988).

1.1.2.1.2.2. Solubility

Hydroxycinnamic acid derivatives have a low but significant solubility in water at neutral or acidic pH. They become more soluble as the pH rises and the hydroxyl groups become ionised increasing the formation of hydrogen bonds. However, attachment of phenolic groups may reduce the water solubility of a polysaccharide. Hydroxycinnamic and benzoic acid derivatives are soluble in many organic solvents such as DMSO, methanol, ethanol, acetone and butan-1-ol. This property can be used to extract phenolics from the aqueous phase into immiscible solvent such as butan-1-ol. By bringing the pH down between 1 and 2, phenolics become uncharged and less soluble in water. By addition of an organic solvent, these phenolics will move to the organic phase in which they are more soluble. The separation of the organic phase from the aqueous phase will allow the isolation of phenolics. However, phenolics cross-linked to polysaccharides will be less tractable to this approach (Fry, 1986).

1.1.2.1.2.3. Stability

The ester linkage between phenolic acid and a polysaccharide is easily hydrolysed by cold sodium hydroxide (Fry, 1982; Fry, 1983). Alkali, used to liberate the esterified phenolics from plant cell wall polymers, renders phenolics highly labile to oxygen especially in the presence of light. Phenolics with two or more hydroxyl groups on the benzene ring (caffeic acid) are particularly susceptible to alkali-induced oxidation and rapidly turn yellow or brown. In addition to triggering oxidation, light can also interconvert the geometrical isomers of phenolic acids. Hydroxycinnamic acid derivatives such as ferulic acids are synthesised as *trans*-isomers, they can be converted to *cis/trans* mixtures upon exposure to light (Fry, 1988).

1.1.2.1.2.4. UV absorbance

Phenolics absorb UV light at wavelengths below 240 nm but also in the 250 - 380 nm range. The absorption spectrum will vary as a function of the chemistry of the phenol (e.g. esterification) and the pH of the solvent (Faulds and Williamson, 1994). Simple phenolics such as tyrosine absorb at about 250 - 280 nm at pH 7 whereas

phenolics with a conjugated side-chain such as cinnamic acid derivatives absorb at longer wavelengths (308 - 340 nm) shifting to 330 - 380 nm on addition of alkali, which induces electron delocalisation (Fry, 1982; Fry, 1988).

1.1.2.1.2.5. UV fluorescence

Many phenolics, when excited by absorption of UV light, re-emit the energy as visible light. This fluorescence can be used to locate and identify phenolics in chromatography or within the cell using UV microscopy to locate phenolics *in-situ*. For example in barley, arabinoxylan compose 71 % of the aleurone cell walls, whereas the starchy endosperm contains 20 % arabinoxylan. Ferulic acid accounts for 0.14 % of the dry weight of a barley grains. Using UV microscopy, about 75 % of the ferulic acids have been located in the outer layers of the kernel (aleurone and husk), in which the arabinoxylans are the most abundant, and about 10 % in the endosperm (Figure 1-5) (Bartolome *et al.*, 1997b). This fluorescence property is also utilised as an empirical method to monitor the enzymatic degradation of the cell wall through the decay of the phenolic autofluorescence. Ferulic acids have been shown to play a key role in maintaining the thermal stability of cell-cell adhesion, and have been used to explain the retention of the texture of Chinese water chestnuts upon cooking (Parker and Waldron, 1995; Waldron *et al.*, 1997). Consistent with this view fluorescence microscopy revealed that the phenolic components are located predominantly at the perimeters of the cell faces (Figure 1-5).

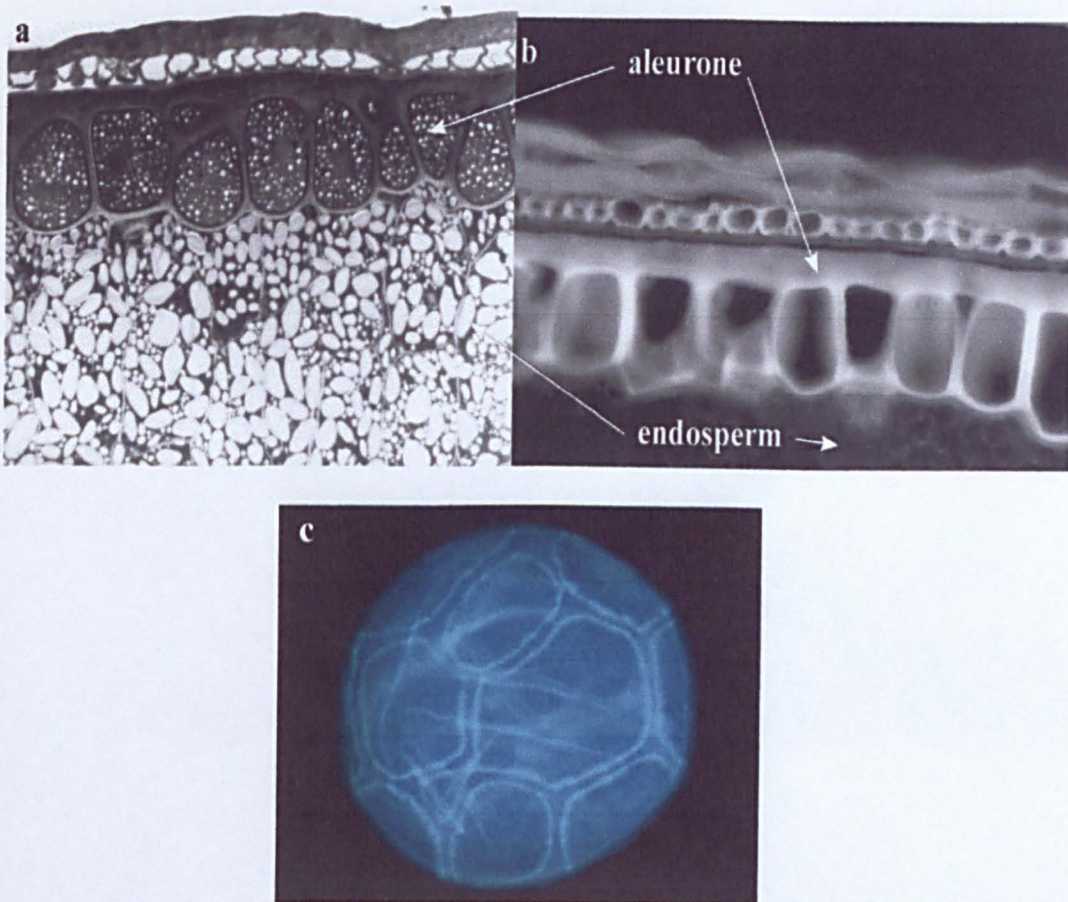


Figure 1-5: (a) Optical microscope image of wheat grain cells, (b) UV autofluorescence image of the same wheat grain cells, (c) fluorescence image of Chinese water chestnut cells (performed by Mary Parker and Keith Waldron, IFR - Norwich).

1.1.2.2. *Dehydrodiferulic acids*

1.1.2.2.1. Covalent cross-linking

The possibility of covalent associations between esterified ferulic acid on wall polysaccharides was realised following the experiment of Geissman and Neukon (Geissman and Neukom, 1971), who showed that aqueous extracts of wheat flour could be made to gel in the presence of hydrogen peroxide and peroxidases, and

demonstrated that a coupling reaction had occurred between two esterified ferulic acid residues attached to arabinoxylan to form dehydrodiferulic acid. However, it was not until Ishii isolated and characterised the “linkage group” from bamboo arabinoxylan that definitive evidence for the existence of the dehydrodiferulic acid ester cross-link was obtained (Figure 1-6) (Ishii, 1991).

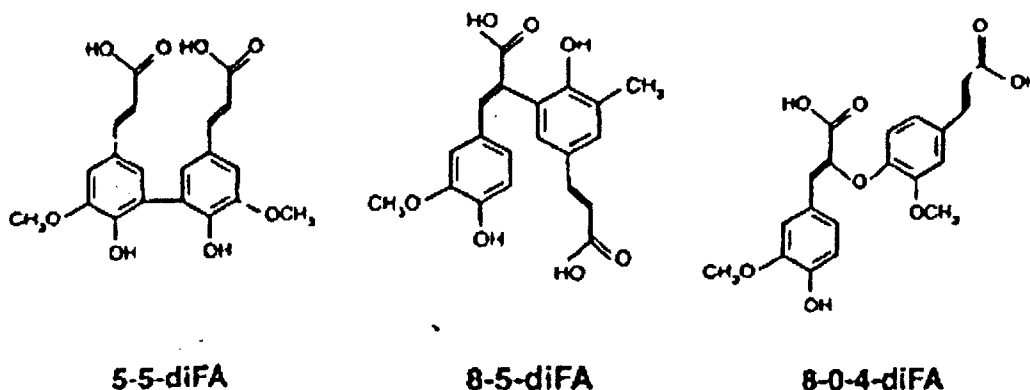


Figure 1-6: Structure of three ferulate dehydrodimers isolated from plant cell walls (Kroon *et al.*, 1999).

To permit the dimerisation of feruloyl ester groups to form cross-links between polysaccharide chains, the feruloyl groups on the same or a different polysaccharide chain must be juxtaposed. Ferulic acids are also believed to cross-link wall polymers to proteins (through tyrosine or cysteine residues) and lignin (Fry, 1986; Hatfield *et al.*, 1999; Iiyama *et al.*, 1994; Kroon and Williamson, 1999). There are two potential methods of covalently coupling ferulates: photoinduced formation of cyclodimers by cycloaddition (2+2) or through enzymatic radical-mediated oxidative coupling producing a range of dehydrodimers (Faulds and Williamson, 1999a; Hatfield *et al.*, 1999; Iiyama *et al.*, 1994; Kroon *et al.*, 1999; Saulnier and Thibault, 1999).

1.1.2.2.2. Influence on the plant cell walls properties

Hydroxycinnamates have an important role in plant cell walls in the maintenance of

integrity, shape and defence against pathogenic ingress. Cross-linking among cell wall polymers can modify its properties such as accessibility, extensibility, plasticity, digestibility and adherence. In the grasses, ferulates play a pivotal role in this cross-linking process, which controls cell wall organisation and structural integrity. These events are carefully controlled by the plant in response to its environment. The degree of cross-linking has also been implicated in the thermal stability of cell-cell adhesion, which is important in the industrial processing of plant cell wall materials. In defence, hydroxycinnamate-derivatives are also important, the presence of ester-linked monomeric and dimeric phenolics leads to a reduction in the biodegradability of the cell wall polysaccharides by ruminant bacterial and fungal enzymes.

1.2. FERULOYL ESTERASES

The plant cell wall is a complex architecture of polysaccharides. For complete hydrolysis of these polysaccharides, microorganisms require a battery of enzymes. Many of these enzymes are induced by factors derived from the growth substrate itself (Faulds *et al.*, 1997). Plant cell wall degradative enzymes have been classified into enzyme systems, including cellulolytic, ligninolytic, xylanolytic and pectinolytic with respect to the general substrate specificities of these enzymes. Plant cell wall polymers can interact with each other to form linked networks. These networks are required for the cell wall rigidity and mechanistic properties. They act as a protective barrier against environmental changes and against the attack of pathogenic organisms (Cosgrove, 2001). Cross-linking of polymers through ferulic acid limits the accessibility of polysaccharidases to their substrates and may hinder degradation by microorganisms. Feruloyl esterases (E.C. 3.1.1.73: FAE, ferulic acid esterase, cinnamoyl esterase, cinnamic acid hydrolase) are a subclass of the carboxylic acid esterases (E.C. 3.1.1.1) that are able to hydrolyse the ester bond between hydroxycinnamic acids and sugars present in the plant cell walls (Williamson *et al.*, 1998b).

1.2.1. Reported feruloyl esterases

Johnson & collaborators first reported ferulic acid esterase activities in 1988 from *Streptomyces* spp. (Johnson *et al.*, 1988a; Johnson *et al.*, 1988b). In recent years, the number of microbial feruloyl esterase activities reported has increased and in particular with the acquisition of related protein sequences in the growing genome databases. Several enzymes have been purified and characterized from aerobic and anaerobic microorganisms that utilise plant cell wall carbohydrates: *Schizophyllum commune* (MacKenzie and Bilous, 1988), *Fibrobacter succinogenes* S85 (McDermid *et al.*, 1990b), *Streptomyces olivochromogenes* (Faulds and Williamson, 1991), *Aspergillus oryzae* (Tenkanen *et al.*, 1991), *Penicillium pinophilum* (Castanares and Wood, 1992), *Neocallimastix* strain MC-2 (Borneman *et al.*, 1992), *Aspergillus niger* (de Vries and Visser, 2001; de Vries *et al.*, 2002b; Faulds and Williamson, 1993; Faulds and Williamson, 1994; Kroon *et al.*, 1996; Williamson *et al.*, 1998a), *Aspergillus awamori* (McCrae *et al.*, 1994), *Talaromyces emersonii* (McCarthy *et al.*, 1994), *Butyrivibrio fibrisolvens* (Dalrymple *et al.*, 1996), *Penicillium expansum* (Donaghy and McKay, 1997), *Bacillus* spp. (Donaghy *et al.*, 1998), *Streptomyces avermitilis* CECT 3339 (Ferreira *et al.*, 1999), *Piromyces equi* (Fillingham *et al.*, 1999), *Clostridium thermocellum* (Blum *et al.*, 2000), *Clostridium stercorarium* (Donaghy *et al.*, 2000), *Penicillium funiculosum* (Kroon *et al.*, 2000). Esterase activities have also been detected in plants and mammalian cells (Andreasen *et al.*, 2001; Sancho *et al.*, 1999).

The gene sequences for a number of these enzymes have been determined, however, the predicted protein sequences would suggest a considerable degree of structural diversity between them. Analysis of the predicted feruloyl esterase protein sequences show that many of these enzymes are modular, comprising of a catalytic domain translationally fused to non-catalytic carbohydrate-binding modules (Coutinho and Henrissat, 1999). Alternatively the catalytic domain may be included in a greater modular complex such as observed for the cellulosome of *Clostridium* spp. (Kroon *et al.*, 2000; Prates *et al.*, 2001; Schubot *et al.*, 2001; Shoham *et al.*, 1999) or for the multiprotein cellulose-binding cellulase-hemicellulase complex from the anaerobic

fungus *Piromyces equi* (Fillingham *et al.*, 1999). Although the first two crystal structures of feruloyl esterase modules have been reported recently from *Clostridium thermocellum* (Prates *et al.*, 2001; Schubot *et al.*, 2001), relatively few studies have been performed to elucidate the functional relationships between sequence diverse feruloyl esterases.

1.2.2. Two types of feruloyl esterase

The two major ferulic acid esterases of *Aspergillus niger* have received most attention, namely FAE-III (AnFAEA, a monomer of 36 kDa designated the A-Type) and CinnAE (AnFAEB, a homodimer of 145 kDa designated the B-Type) (de Vries *et al.*, 2002b; Faulds and Williamson, 1993; Faulds and Williamson, 1994; Kroon *et al.*, 1996; Ralet *et al.*, 1994b). Their production is induced by growth on complex plant cell wall polysaccharides (e.g. wheat bran for AnFAEA, sugar beet pulp for AnFAEB) and no activity is detected when the fungus is grown on glucose. The enzymes are secreted into the culture supernatant during growth on these substrates.

These enzymes have been classified as either Types-A or B dependent on their substrate specificity for aromatic moieties (Kroon *et al.*, 1999). The enzymes show a preference for the phenolic linkage to the primary sugar and vary in their ability to release dehydrodiferulic acids from esterified substrates (de Vries and Visser, 2001; Fillingham *et al.*, 1999; Kroon *et al.*, 1997a; Kroon *et al.*, 1996; Kroon *et al.*, 1999; Kroon *et al.*, 2000). All esterases show acetyl xylan esterase activity regardless of whether they are assigned as acetyl or feruloyl esterases. However, the specific acetyl xylan esterase activity is rather low for the enzymes classified as feruloyl esterases. The feruloyl esterase substrate specificity for either methyl sinapate or methyl caffeate is the basis of this putative classification.

1.2.2.1. Type-A feruloyl esterase

Type-A feruloyl esterases tend to be induced during growth on cereal-derived substrates. The enzyme releases ferulic acid from 1,5 ester-linked feruloylated arabinose (Figure 1-4) (Ralet *et al.*, 1994b), and is also able to release low quantities

of 5,5' and 8-*O*-4' ferulate dehydrodimers from plant material when pre-treated with a xylanase or when a xylanase was co-incubated with the feruloyl esterase (Figure 1-6) (Bartolome *et al.*, 1997a; Faulds and Williamson, 1995; Kroon *et al.*, 1999; Williamson *et al.*, 1998a). There is no report of this enzyme releasing diferulate from pectin-derived material. Type-A feruloyl esterases also show a preference for the phenolic moiety of the substrate that contains methoxy substitutions, especially at the carbon 3 and/or 5, as occurs in ferulic and sinapic acids (Figure 1-3). Regarding the specificity against synthetic substrates, Type-A feruloyl esterases are active against methyl ferulate, methyl sinapate and methyl *p*-coumarate but not methyl caffeate. They appear to prefer hydrophobic substrates with bulky substituents on the benzene ring (Table 1-3) (Kroon *et al.*, 1997a; Kroon *et al.*, 1999; Williamson *et al.*, 1998a).

1.2.2.2. *Type-B feruloyl esterase*

Type-B feruloyl esterases are preferentially secreted by growth on sugar beet pulp. The enzyme released ferulic acid ester-linked to either C-2 of feruloylated arabinose or C-6 of feruloylated galactose residues (Figure 1-4) (Kroon and Williamson, 1996) but is unable to release the dimeric forms of ferulate. This enzyme also works on 1,5-linked ferulic acid as found in cereals but at a lower rate than the Type-A esterases (Kroon *et al.*, 1996; Ralet *et al.*, 1994b; Williamson *et al.*, 1998a). The Type-B feruloyl esterases show a preference for the phenolic moiety of the substrate that contains one or two hydroxyl substitutions, as found in *p*-coumaric and caffeic acids respectively. Hydrolytic rates are significantly lowered when a methoxy group is present. Regarding the specificity against synthetic substrates, Type-B feruloyl esterases are active against methyl caffeate, methyl ferulate and methyl *p*-coumarate but not methyl sinapate (Table 1-3).

Table 1-3: Substrate specificities of feruloyl esterases from *Aspergillus niger* (Faulds and Williamson, 1993^a; Kroon *et al.*, 1999^b; Ralet *et al.*, 1994b^c).

Substrates	Type-A:	Type-B:
	FAEA (FAE-III)	CinnAE (FAEB)
<u>Activity on methyl esters^a</u>		
MFA (Methyl Ferulate)	Y	Y
MSA (Methyl sinapate)	Y	N
MCA (Methyl caffeate)	N	Y
MpCA (Methyl <i>p</i> -coumarate)	Very low	Y
<u>Activity on feruloylated oligosaccharides^c</u>		
FAXX (O-[5-O-(<i>trans</i> -feruloyl)- α -L-arabinofuranosyl]-(1-3)-O- β -D-xylopyranosyl-(1-4)-D-xylopyranose) found in wheat bran.	Y	Y
Ara ₂ F (O-[2-O-(<i>trans</i> -feruloyl)- α -L-arabinofuranosyl]-(1-5)-L-arabinofuranose) found in sugar beet pulp.	N	Y
<u>Ability to release dehydrodimers^b</u>	Y	N

Y= yes, N= no.

1.2.2.3. Substituted phenylalkanoate methyl esters highlight different active site properties for the Type-A and B esterases

Structural studies and the use of serine-specific protease inhibitors, such as diisopropylfluorophosphate (DFP) and aminoethylbenzenesulphonylfluoride (AEBSF), have established that specific serine residues are involved in the catalytic reaction of many functionally diverse hydrolytic enzymes, including proteases (Blow

et al., 1969), triacylglycerol lipases (Brady *et al.*, 1990; Winkler *et al.*, 1990), acetylcholinesterases (Sussman *et al.*, 1988) and feruloyl esterases (Aliwan and Williamson, 1998; Fillingham *et al.*, 1999; Kroon *et al.*, 1996; Kroon *et al.*, 2000; Prates *et al.*, 2001; Schubot *et al.*, 2001). Despite evidence for a common catalytic mechanism these hydrolytic enzymes display unique substrate specificities and substrates binding properties.

1.2.2.3.1. Specificity for substituted phenylalkanoate methyl esters

In 1997, Kroon & collaborators determined the substrate specificity of AnFAEB and AnFAEA, using a wide range of methyl esters of phenolic acids (Kroon *et al.*, 1997a). The substrates tested vary in the nature of the substitution groups on the benzene ring and by the length of the aliphatic chain. Neither enzyme shows activity for substrates with only one or two carbon atoms in the aliphatic chain nor is activity detected against substrates with five carbon atoms in the aliphatic chain. These results indicate that there is a distance requirement between the aromatic ring and the ester bond of the substrate to enable hydrolysis by these esterases. The authors calculated the linear distances between the centre of the benzene ring and the ester bond oxygen in energy-minimised conformation for several substrates. They determined that both esterases required the distance to be greater than 4 Å but less than 8 Å. The two esterases were complementary in their specificities for substitutions on the benzene ring, suggesting that substrate acceptability was governed by these substitutions to permit alignment of the substrate ester bond at their respective active sites (Williamson *et al.*, 1998a). Out of 19 compounds tested only two were substrates for both AnFAEB and AnFAEA indicating that the active sites of these enzymes are likely to contain structural differences.

1.2.2.3.2. Inhibition by substrate analogs and potential inhibitors

Substrate analogs such as methyl phenylalkanoate esters, which are not hydrolysed by feruloyl esterase, or products such as phenylalkanoic acids were utilised by Kroon & collaborators as potential inhibitors (Kroon *et al.*, 1997a). All three phenylalkanoate methyl esters tested behaved as competitive inhibitors of AnFAEA,

able to bind to the free enzyme but not to the enzyme-substrate complex. The presence of inhibitors reduces the enzymes specificity and therefore increases the apparent K_m with no change in the apparent k_{cat} . However, the K_i and K_m values are similar suggesting that AnFAEA exhibits a non-specific hydrophobic binding site with affinity for the benzene ring of these substrates. For AnFAEB, all three phenylalkanoate methyl esters tested behaved as mixed non-competitive inhibitors, which combined the effects of competitive and non-competitive inhibition. Substrates analogs are able to bind either the free enzyme or the enzyme-substrate complex forming a ternary enzyme-substrate-inhibitor complex, which is catalytically inactive. By consequence, both specificity and catalytic properties of the enzyme are modified and results in an increase of the apparent K_m and in a decrease of the catalytic rate (k_{cat}). Three phenylalkanoic acids were also tested as inhibitors of AnFAEB. With two of the acids, the inhibition is mixed non-competitive, which means that the dissociation constants for combination with both free enzyme and enzyme-substrate complex are different. With the third acid, the inhibition is simple non-competitive, which means that the inhibitor can bind both enzyme and enzyme-substrate complex with the same association rate. The inhibition data tend to confirm that the active site of AnFAEA and AnFAEB are significantly different, with different behaviours toward substrate analogs and inhibitors.

1.2.3. A third type of esterase

A third type of esterase with broad specificity against synthetic hydroxycinnamic acids (ferulic, caffeic, *p*-coumaric and sinapic) has been reported as an acetyl esterase (XYLD) from *Pseudomonas fluorescens* subsp. *cellulosa* (Ferreira *et al.*, 1993). The data indicate that XYLD is able to cleave ester linkages in plant cell walls, although the enzyme exhibits greatest activity towards the release of acetic acid from 4-nitrophenyl acetate. The hydrolysis rate of XYLD esterase for hydroxycinnamic acids as well as for acetylated substrates is significantly lower (25-500 folds) than reported for the characterised fungal feruloyl esterases (de Vries *et al.*, 2002b; Faulds and Williamson, 1994; Kroon *et al.*, 1996; Kroon *et al.*, 2000) and other microbial acetyl xylan esterases (Basaran and Hang, 2000; Blum *et al.*, 1999; Degrassi *et al.*, 2000; Linden *et al.*, 1994; McDermid *et al.*, 1990a). In addition,

XYLD is capable of releasing 5-5' ferulic dehydrodimers from barley and wheat cell walls (Bartolome *et al.*, 1997a). In respect of this broad substrate specificity, XYLD has been tentatively classified as a "general plant cell wall esterase".

1.2.4. Feruloyl esterases are subject to complex regulation

The expression of many fungal genes coding for cell wall degrading enzymes are subject to regulation by carbon catabolic repression, as exercised by the carbon catabolite repressor CreA from *A. niger* and *A. nidulans* (de Vries *et al.*, 2002a; de Vries *et al.*, 1999b; Dowzer and Kelly, 1991; Orejas *et al.*, 2001; Ruijter and Visser, 1997), Cre-1 from *N. crassa* (Ebbole, 1998), or Cre1 from *T. reesei* (Strauss *et al.*, 1995). Cell wall degrading enzymes are also specifically induced via the xylanolytic transcriptional activator XlnR (de Vries and Visser, 1999; van Peij *et al.*, 1998b), in addition to positive or negative regulation by factors derived from the growth substrates themselves (de Vries *et al.*, 2002b; Faulds *et al.*, 1997; Faulds and Williamson, 1999b).

1.2.4.1. Influence of ferulic acid on AnfaeA expression

In 1999, de Vries & collaborators studied the induction of AnfaeA gene by polymeric substrates (de Vries *et al.*, 1999a). Using an *faeA* multicopy transformant of *A. niger* (NW154::pIM3207.7) the production of feruloyl esterase was monitored on minimum medium containing different polymeric carbon sources after 6 days of growth (de Vries *et al.*, 1997). Growth on crude substrates resulted in a greater production of extracellular AnFAEA than growth on xylan or pectin, possibly due to the presence of compounds that are more easily metabolised at the early stages of culture with these crude materials resulting in overall better growth. However, the addition of ferulic acid to oat spelt xylan medium enhanced the extracellular AnFAEA activity, reaching levels greater than on the crude substrate alone (de Vries *et al.*, 1999a; Faulds *et al.*, 1997; Faulds and Williamson, 1999b). Since oat spelt xylan does not contain detectable levels of ferulic acid, this suggested the presence of two systems for the induction of AnfaeA: specific induction by ferulic acid present in the growth medium and induction through the xylan related factor XlnR, which

influences a large number of xylanolytic and cellulolytic genes of which *AnfaeA* is among them (de Vries *et al.*, 2002b; Gielkens *et al.*, 1999; van Peij *et al.*, 1998a). Consistent with the presence of two induction systems was the poorer expression of *AnfaeA* during growth on pectin as compared with xylan. In the absence of xylan low quantities of ferulic acid present in pectin could be responsible for the induction of *AnfaeA*. To investigate further the effect of ferulic acid on the *AnfaeA* induction, different concentrations of ferulic acid were added to *A. niger* oat spelt xylan growth medium. When 0.01 % ferulic acid is added to the medium, a strong increase in FAE activity was observed. The FAE activity accumulates slower when 0.03 % ferulic acid is supplemented. These data indicate that ferulic acid assists the induction of *AnfaeA* on xylan. Higher ferulic acid concentrations, however, result in a slower response, likely due to toxic effects of ferulic acid internalisation into the cell, which is required for *faeA* specific induction (de Vries *et al.*, 1999a; Faulds *et al.*, 1997). To summarise, induction on pectin and enhanced induction on xylan require the presence of ferulic acids. In contrast, xylanase activity can be suppressed by the addition of ferulic acid, indicating that the regulatory mechanisms involved are likely to be multi-faceted and interactive in the production of feruloyl esterases and xylanases (Faulds *et al.*, 1997; Faulds and Williamson, 1999b).

1.2.4.2. Regulation of *AnfaeA* by the xylanolytic transcriptional activator *XlnR*

The transcriptional activator *XlnR* of *A. niger* (van Peij *et al.*, 1998b) directs the expression of certain cellulolytic genes (Gielkens *et al.*, 1999; van Peij *et al.*, 1998a) as well as genes encoding xylanolytic enzymes such as endoxylanases (de Graaff *et al.*, 1994), β -xylosidase (van Peij *et al.*, 1998b), α -glucuronidase (van Peij *et al.*, 1998a), feruloyl esterase (de Vries *et al.*, 1997) and acetyl xylan esterase (de Graaff *et al.*, 1992). *XlnR* is a member of the GAL4 family of transcription factors, binding DNA at the consensus nucleotide site 5'-GGCTAA-3' via a zinc binuclear cluster ($\text{Zn(II)}_2\text{Cys}_6$) (van Peij *et al.*, 1998a). While most zinc binuclear cluster proteins have a dimeric nature and bind to symmetric sites, the mode of binding of *XlnR* is not known (van Peij *et al.*, 1998b).

In the presence of xylose or xylan, XlnR loss-of-function mutants are unable to induce either the transcription of genes encoding xylanolytic enzymes or the transcription of accessory enzyme encoding genes such as those of the feruloyl esterases (van Peij *et al.*, 1998a). The inability to degrade xylan probably affects the release of inducers from polymeric substrates, such as ferulic acids, and therefore, the absence of phenolic compounds may prevent any subsequent induction pathway.

1.2.4.3. Carbon catabolite repression of *AnfaeA*

In the presence of preferred substrates such as glucose and fructose the carbon catabolic repressor CreA prevents transcription of genes encoding enzymes involved in the nutritional assimilation of carbon including those involved in the degradation of plant cell wall polymers (Ruijter and Visser, 1997). This carbon catabolic repressor CreA is a wide-domain repressor that has been shown to function by binding to DNA targets conforming to the consensus sequence 5'-SYGGRG-3' (S, Y, and R represent C or G, C or T, and A or G, respectively) via a pair of zinc fingers of the Cys₂-His₂ class. CreA homologues have also been found in other filamentous fungi such as *Aspergillus nidulans* (CreA), *Trichoderma reesei* (Cre1) and *Neurospora crassa* (Cre-1) as well as in the yeast *Saccharomyces cerevisiae* (Mig1p) (de la Serna *et al.*, 1999; Ebbole, 1998; Gancedo, 1998; Orejas *et al.*, 2001; Shroff *et al.*, 1997; Strauss *et al.*, 1995).

The *faeA* gene was found to be expressed in the presence of xylose, xylan and ferulic acid at similar levels. However, the addition of glucose strongly reduces the expression of *faeA* in presence of xylose and eliminates the expression of *faeA* in presence of xylan and ferulic acid. Whereas when using an *A. niger creA* mutant, *faeA* did not suffer the same level of repression in presence of glucose (de Vries *et al.*, 2002b; de Vries and Visser, 1999). Xylose concentrations influence XlnR-induced expression of *faeA*. *AnfaeA* expression is increased in the presence of xylose but is greater in presence of limiting xylose concentrations (0.03 %) than with substrate levels of xylose (1 %). This phenomenon has been explained by modulation of gene expression via CreA (de Vries *et al.*, 1999b). Xylose simultaneously induces the xylan-dependent XlnR system and causes carbon catabolic repression,

mediated by CreA. In parallel the expression level of xylanolytic genes on xylan is usually higher than on xylose. Xylose is released gradually from xylan, resulting in a low xylose concentration in the medium. Under these conditions, repression by xylose mediated by CreA is low, and high *faeA* expression levels are detected. Therefore, the actual level of expression is influenced by the balance between induction by XlnR and repression by CreA. Both effects would depend on the number and position of the consensus sequences they bind, 5'-SYGGRG-3' (Kulmburg *et al.*, 1993; Orejas *et al.*, 2001) and 5'-GGCTAA-3' (van Peij *et al.*, 1998a) for CreA and XlnR respectively, in the promoter DNA sequences of the xylanolytic genes. However, not all enzymes involved in the degradation of cell wall polymers are repressed in presence of glucose. Actually, some xylanase activity can be detected when *A. niger* is grown on glucose medium suggesting that not all xylanolytic genes are under the control of the catabolic repressor CreA (Faulds and Williamson, 1994).

1.2.5. Synergy between feruloyl esterases and plant cell wall degrading enzymes

The esterases act to enable and facilitate the access of hydrolases to the backbone wall polymers. Most of the FAEs have been shown to act synergistically with xylanases, cellulases and pectinases to breakdown complex plant cell wall carbohydrates (Faulds *et al.*, 1995a; Faulds *et al.*, 1995b; Faulds and Williamson, 1991; Faulds and Williamson, 1993; Faulds and Williamson, 1994; Ferreira *et al.*, 1993; Fillingham *et al.*, 1999; Garcia-Conesa *et al.*, 1998; Kroon *et al.*, 1999; Kroon and Williamson, 1996; Ralet *et al.*, 1994b; Yu *et al.*, 2002).

In 1995, Faulds & Williamson reported that AnFAEA from *A. niger* was able to release up to 95 % of the alkali-extractable ferulic acids from wheat bran, in 5 hours, in the presence of a xylanase from *Trichoderma viride* (Faulds and Williamson, 1993; Faulds and Williamson, 1995). However, it was subsequently shown that the source of the xylanase utilised in these extractions is important, as less ferulic acid was released by AnFAEA in the presence of xylanases from either *A. niger* or *P. fluorescens*, when compared with the *Trichoderma viride* xylanase (Bartolome *et*

al., 1995). The source of the plant cell wall material and the xylanase applied will also influence the release of ferulic acid. For example, with Brewer's spent grain, the combination of AnFAEA and *T. viride* xylanase could only release 30 % of the available ferulic acids compared to the 95 % released from wheat bran (Bartolome *et al.*, 1997b).

de Vries & collaborators studied the ability of 15 xylanolytic and pectinolytic enzymes from *Aspergillus niger* to degrade wheat arabinoxylan or sugar beet pectin (SBP) either as individual enzymes or in concert (de Vries *et al.*, 2000). These studies examined the simultaneous and sequential action of main-chain cleaving and accessory enzymes, using intact or pre-treated material with endoxylanases (wheat arabinoxylan) or rhamnogalacturonases (sugar beet pectin). These data demonstrated that there is a complex relationship between the xylanolytic enzymes (α -glucuronidase, *endo*-xylanase, α -galactosidase, arabino-furanohydrolase, β -xylosidase, AnFAEA) that act to synergistically degrade water insoluble pentosan (WIP) from wheat flour. Given the majority of these enzymes are expressed in the presence of xylose and are under the control of the xylanolytic transcriptional activator XlnR, it would seem they have evolved to maximise the use of saprophytic substrates (de Vries *et al.*, 1999a; de Vries and Visser, 1999; de Vries *et al.*, 1999b; Gielkens *et al.*, 1999; van Peij *et al.*, 1998a).

AnFAEA activity can be detected against SBP but only in the presence of rhamnogalacturonases, showing that the association of these two enzymes is essential to release galactose-linked ferulic acids from SBP (de Vries *et al.*, 2000; Ralet *et al.*, 1994b). The arabinose-linked ferulic acids from SBP however, are released by the second feruloyl esterase from *A. niger* (CinnAE, AnFAEB), which acts synergistically with an *A. niger* α -L-arabinofuranosidase, to remove arabinose substitutions from the arabinan main chain. Further, the *A. niger* *endo*-arabinase will cleave the arabinan main chain and release feruloylated arabinose oligosaccharides, which may then be hydrolysed by AnFAEB (de Vries *et al.*, 2000; Kroon and Williamson, 1996). AnFAEA and AnFAEB clearly have complementary activities in terms of their ability to degrade plant cell wall materials. However, to maximise the

effects of the feruloyl esterases, they need to be associated with specific carbohydrate hydrolases in order to facilitate the access to their required substrates.

1.2.6. Structure and catalytic mechanism of feruloyl esterases

Structural and sequence comparisons of enzyme families show that catalytic groups have distinctive patterns of variation and that the proteins themselves sometimes have unexpected evolutionary and structural relationships with other families that have completely different functions (Dodson and Wlodawer, 1998). Feruloyl esterases are part of the serine esterase family. There is little sequence identity between the Type-A and B feruloyl esterases but they do show distant relationships with other enzymes. FAEA from *A. niger* shows 32 - 36 % identity with a group of fungal lipases (Aliwan *et al.*, 1999). Whereas, Type-B feruloyl esterases from *P. funiculosum* (Kroon *et al.*, 2000) and *A. niger* (de Vries *et al.*, 2002b), show similarity with acetyl xylan esterases (44 - 46 %) and tannases (23 - 26 %). These similarities probably arise from the conservation of the main structural folds that relate these enzyme classes.

1.2.6.1. Structure of feruloyl esterases

The first X-ray crystallographic structure of feruloyl esterase modules was reported for xylanase 10B (Prates *et al.*, 2001) and xylanase Z (Schubot *et al.*, 2001) from *Clostridium thermocellum*. The feruloyl esterase structure exhibits a α/β hydrolase fold characteristic of a number of hydrolytic enzymes with a Ser-His-Asp catalytic triad at their active site.

1.2.6.1.1. The α/β hydrolase fold

Enzymes as diverse as haloalkane dehalogenase (Franken *et al.*, 1991), acetylcholinesterase (Sussman *et al.*, 1988), diene lactone hydrolase (Pathak and Ollis, 1990), and serine carboxypeptidase (Liao *et al.*, 1992), share a similar tertiary structure. All catalyse hydrolysis reactions and feature a common fold, which has been termed the α/β hydrolase fold (Ollis *et al.*, 1992). The canonical α/β hydrolase

fold consists of a central core, mostly parallel β sheet of eight strands with every second strand configured antiparallel to the former (Figure 1-7). The parallel strands $\beta 3$ to $\beta 8$ are connected by α helices, which pack on either side of the central β sheet. This eight-stranded α/β -fold is also called the TIM-barrel and forms the active site cleft at the end of the α/β -fold structure, between $\beta 5$ and $\beta 8$ strands (Schubot *et al.*, 2001). The β sheet has a left-handed superhelical twist such that the surface of the sheet covers about half a cylinder and the first and the last strands cross each other at an angle of $\sim 90^\circ$. The curvature of the β sheet may differ significantly among the various enzymes, and also, the spatial position of the α helices may vary considerably (Jaeger *et al.*, 1999).

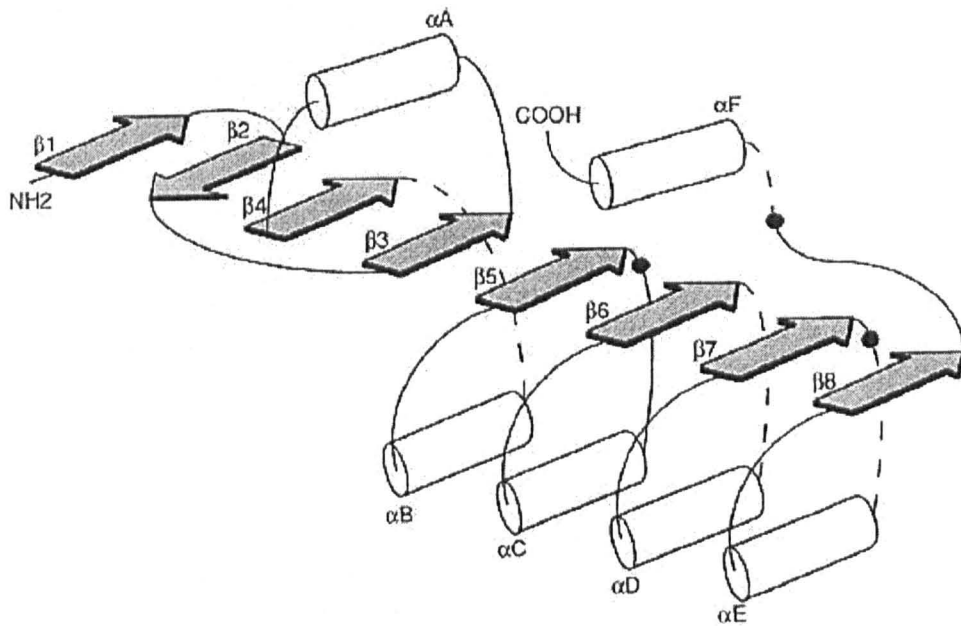


Figure 1-7: Canonical fold of α/β hydrolases. α helices are indicated by cylinders, and β strands are indicated by shaded arrows. The topological position of the active-site residues is shown by solid circle; the nucleophile is the residue after β strand 5, the Asp/Glu residue is after β strand 7, and the histidine residue is in the loop between $\beta 8$ and αF (from Jaeger *et al.*, 1999).

The FAE_XynZ domain displays a α/β - topology with the eight-stranded sheet (Figure 1-8) assuming an open conformation and the helices being positioned on both sides (Schubot *et al.*, 2001). FAE_Xyn10B is a compact globular protein. The overall topology of the protein is a classical α/β -hydrolase fold based on an eight-stranded β sheet surrounded by α helices, where six of the strands are parallel with a pair of antiparallel strands at the C-terminal side of the fold (Figure 1-9) (Prates *et al.*, 2001).

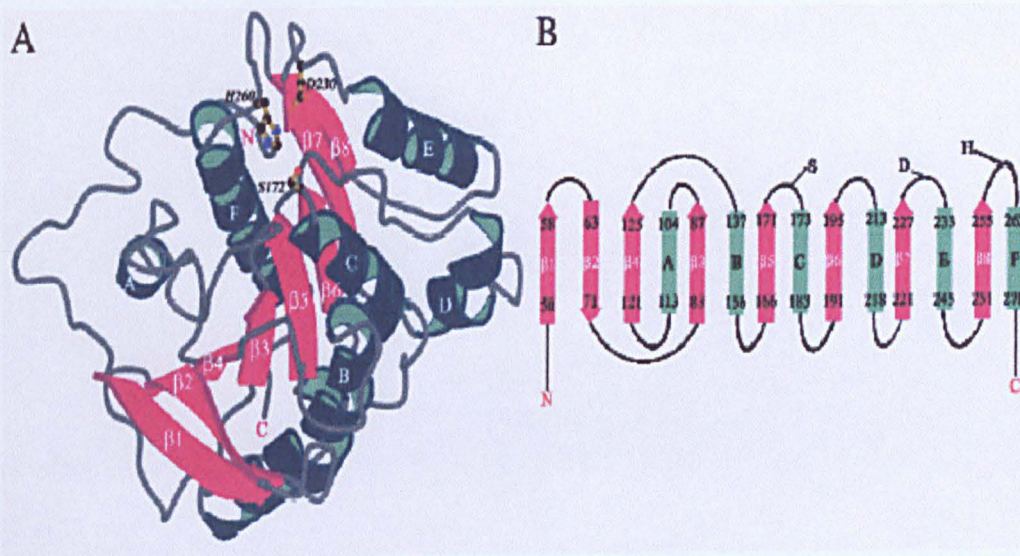


Figure 1-8: (A) Ribbon drawing of the FAE_XynZ backbone structure showing the α/β domain with the twisted sheet at the centre. Also shown are the residues that constitute the catalytic triad. (B) Topology plot of FAE_XynZ with the helices displayed as blue cylinders and the strands as pink arrows. The position of the catalytic residues at the C-terminal end of the β -sheet are provided (from Schubot *et al.*, 2001).

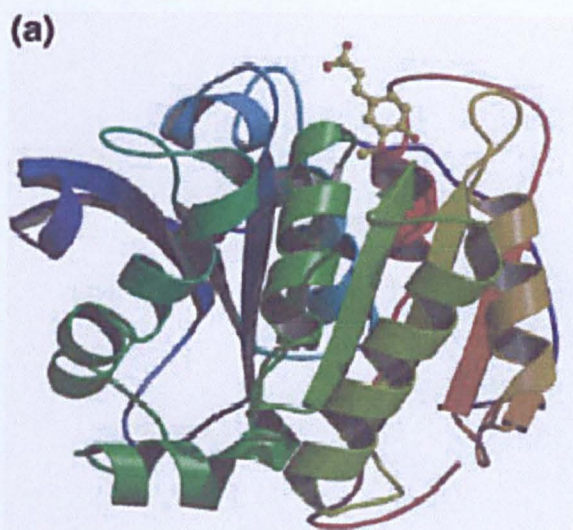


Figure 1-9: Ribbon diagram of the (S954A) FAE_Xyn10B structure with the location of the bound ferulate in “ball-and-stick” representation (from Prates *et al.*, 2001).

1.2.6.1.2. The catalytic residues are located in the “nucleophilic elbow”

The active site of the α/β hydrolase fold enzymes consist of three catalytic residues; a nucleophile residue (serine, cysteine, or aspartate), a catalytic acid residue (aspartate or glutamate) and a histidine residue (Dodson and Wlodawer, 1998). The nucleophilic serine is located at the centre of a universally conserved Gly-X-Ser-X-Gly pentapeptide, which forms a sharp γ -like turn between the β_5 strand of the canonical α/β hydrolase central β sheet and the following α helix C. The γ turn is characterised by energetically unfavourable main chain ϕ (rotation of N-C bonds)

and ψ (rotation of C-C bonds) torsion angles of the nucleophile. Because of the steric restrictions imposed by the tight turn structure, the residues located two positions before, and two positions behind the nucleophile must be small, often glycine residues. This strand-nucleophile-helix arrangement forms the most conserved structural feature of the α/β hydrolase fold and is called the “nucleophilic elbow”: a tight turn with the catalytic serine residue sitting at the top (Schubot *et al.*, 2001). The nucleophilic elbow positions the nucleophilic residue to allow easy access on one side by the active site histidine residue and on the other by the substrate. The sharp turn also positions the nucleophile at the N-terminal end of the following helix, thereby helping to stabilise the tetrahedral intermediate and the ionised form of the nucleophile formed during the catalytic process. Since the sequence homology among α/β hydrolases is generally low, despite their shared structural elements, the nucleophilic elbow has become the fingerprint feature commonly used to identify proteins of this enzyme family based on their primary structure alone (Schrage and Cygler, 1997).

1.2.6.2. *Catalytic mechanism of feruloyl esterases*

The first protease catalytic site determined 30 years ago by Blow & colleagues (Matthews *et al.*, 1967), was that of the α -chymotrypsin. Their analysis showed that Ser195 and His57 residues were directly involved in catalysis. Examination of the X-ray crystallographic structure revealed that the catalytic serine residue (Ser195) was near enough to His57 to form a hydrogen bond and that aspartate residue 102 was hydrogen-bonded to His57, and could form a salt bridge with this histidine residue. The conservation of this aspartate residue in related sequences showed that this residue was an important component of the catalytic structure, which established the Ser-His-Asp triad.

Feruloyl esterases are hydrolases acting on the ester bond between cell wall polysaccharide and ferulic acid. Their structure exhibits a α/β hydrolase fold pattern, which is common to a number of hydrolytic enzymes, with a Ser-His-Asp catalytic triad at their active site. This catalytic triad is similar to that observed for other α/β -

hydrolase superfamily members (Schrage and Cygler, 1997), such as serine proteases and lipases (Blow *et al.*, 1969; Brady *et al.*, 1990; Schrage *et al.*, 1991; Winkler *et al.*, 1990). Even though different members of this group target a large variety of substrates, the general mechanism of hydrolysis is believed to be conserved.

1.2.6.2.1. Hydrolysis of the substrate

The activation of the nucleophilic serine occurs through a charge-relay system, which involves the catalytic histidine and aspartic acid residues. After activation the nucleophilic serine residue attacks the substrate carbonyl carbon, leading to the formation of a transient tetrahedral intermediate, which is characterised by a negative charge on the carbonyl oxygen atom of the ester bond and four atoms bonded to the carbonyl carbon atom arranged as a tetrahedron (Figure 1-10) (Warshel *et al.*, 1989). This intermediate is stabilised by hydrogen bonds between the negatively charged carbonyl oxygen atom (the “oxyanion”) and two main-chain NH groups (the “oxyanion hole”), while the positively charged histidine is stabilised by a hydrogen bond to the catalytic acid. At this stage the acid component of the substrate is esterified to the nucleophilic serine to form the “covalent intermediate” or “acyl enzyme”, whereas the alcohol component diffuses away (Verschuere *et al.*, 1993). The next stage is a two-step hydrolysis of the covalent intermediate involving a water molecule. The water molecule is activated through the charge-relay system leading to the attack by OH^- ion of the carbonyl carbon atom of the acyl group covalently bound to the catalytic serine residue, resulting in the formation of a tetrahedral intermediate stabilised by interactions with the oxyanion hole. After proton transfer, the acyl component is released and the enzyme can start a new hydrolysis reaction (Blow *et al.*, 1969; Brady *et al.*, 1990; Jaeger *et al.*, 1999; Prates *et al.*, 2001; Schubot *et al.*, 2001; Winkler *et al.*, 1990).

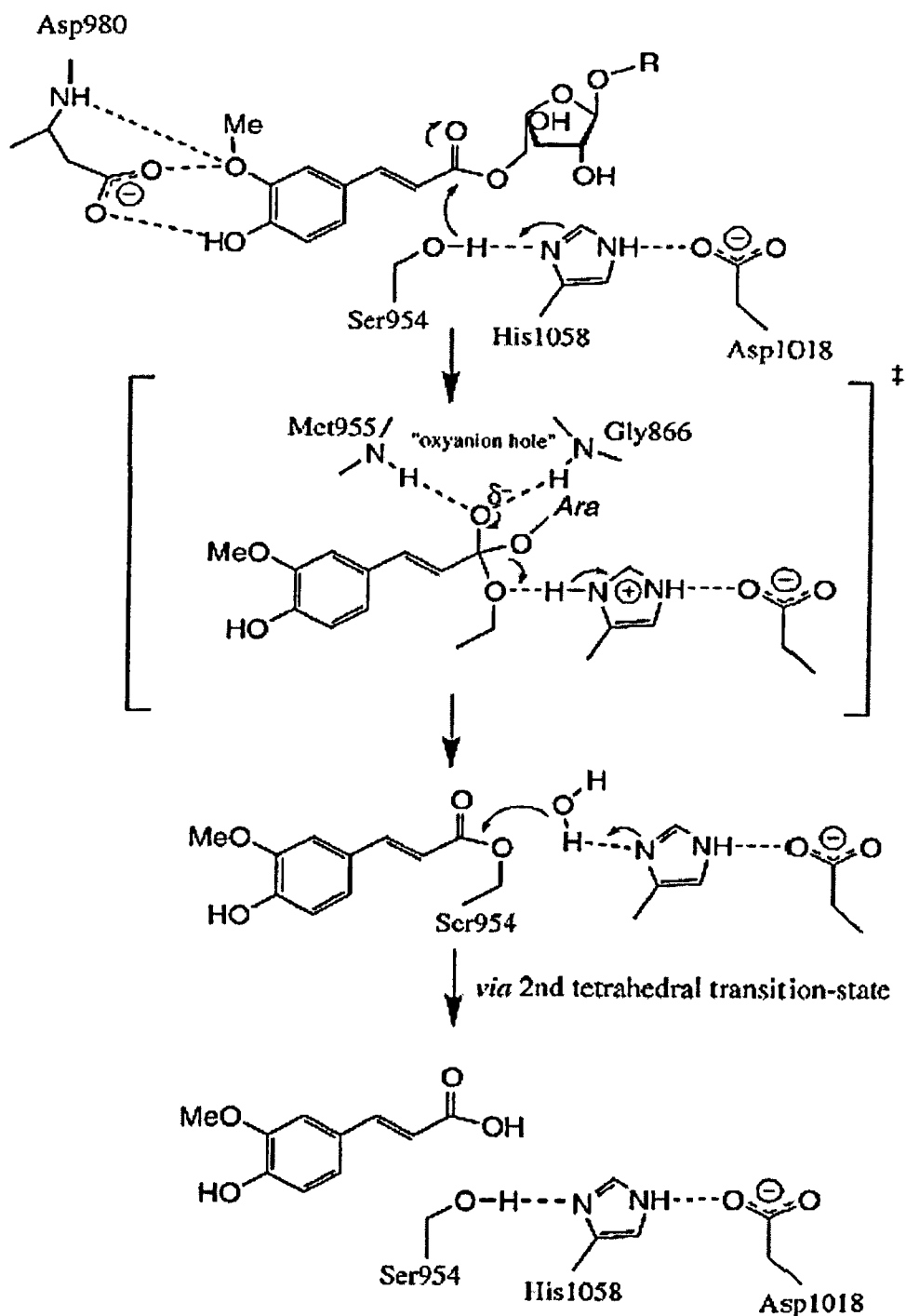


Figure 1-10: Catalytic mechanism of a feruloyl esterase. Catalysis is performed via the formation and subsequent breakdown of a covalent acyl-enzyme intermediate via tetrahedral transition states sometimes known as the “tetrahedral intermediates”. In the case of the Xyn10B FAE, the main-chain amide hydrogens of Gly866 and Met955 provide stabilization of the developing negative charge in the “oxanyan hole” (from Prates *et al.*, 2001).

1.2.6.2.2. Substrate binding site in feruloyl esterases

For FAE_XynZ from *C. thermocellum*, the aromatic part of the substrate has been shown to form hydrogen bonds with the nucleophilic serine (Ser172) and the catalytic histidine (His260) as well as with the NH-groups of isoleucine 90 and methionine 173 residues that constitute the oxyanion hole. The ferulic acid binding site is located in a hydrophobic pocket created by the side chains of residues Ile90, Met173, Ala198, Pro199 and Leu232. The open geometry of the hydrophobic pocket and its exposure to the solvent suggest that the enzyme belongs to the Type-A feruloyl esterases that are able to accommodate bulky substituent such as diferulates at their active site (Kroon *et al.*, 1997a; Williamson *et al.*, 1998a). Moreover, of the residues that form the ferulic acid binding pocket, other than the catalytic triad, Met173 and Leu232 are conserved in FAE_XynY from *C. thermocellum* and FAE A from *Orpinomyces* sp. (Blum *et al.*, 2000). Next to the ferulic acid binding site, a hydrophobic pocket is formed to interact with the carbohydrate part of the substrate. Of the residues composing this hydrophobic pocket, His88, Trp97, Leu171, Phe262, and Trp265 are strictly conserved between FAE_XynZ and FAE A from *Orpinomyces* sp., suggesting similar binding interactions of both enzymes with the carbohydrate part of the substrate (Schubot *et al.*, 2001).

The 1.4 Å resolution structure of FAE_Xyn10B from *C. thermocellum* (Figure 1-9) reveals that the ferulic acid binds in a shallow pocket on the surface of the enzyme, which provides hydrophobic and hydrogen interactions with the methoxy and hydroxy substituents of ferulic acid. The size and the shape of the whole surface cleft suggest that a ferulate cross-bridge could not be accommodated into the substrate binding pocket, forming a more compact structure compared to FAE_XynZ (Figure 1-8). However, the structure did not reveal any binding site for the sugar moieties, suggesting that the enzyme has evolved to recognise the ferulate moiety with high specificity while displaying more relaxed specificity toward the sugar units, probably in order to accommodate the large range of ferulated xylans found in the plant cell wall (Prates *et al.*, 2001).

Despite belonging to the α/β -hydrolase superfamily and retaining a common catalytic mechanism, these feruloyl esterases display some very different structural properties resulting in disparate substrate binding specificities and therefore disparate enzyme properties.

1.2.7. Industrial applications of feruloyl esterases

Feruloyl esterases were discovered relatively recently (Johnson *et al.*, 1988b), and constitute an interesting group of enzymes that have the potential for use over a broad range of applications in the agri-food industries. There is considerable scope to utilise feruloyl esterases to process otherwise waste agricultural materials into valuable products. As a consequence these enzymes have become a focus for research to identify and produce enzymes capable of acting on a diverse range of substrates at levels commensurate with their applications to commercial processes (Juge *et al.*, 2001).

1.2.7.1. Bread making

Bakery enzymes are regarded as processing aids, since they are deactivated by high temperature at an early stage of the baking process. Ferulic acids are postulated to form cross-links with proteins in wheat (Hoseney and Faubion, 1981; Iiyama *et al.*, 1994), which are important to the rheology of dough, and consequently may play a role in the baking process. Enzymes such as feruloyl esterases, arabinofuranosidases, arabinofuranohydrolases and *endo*-xylanases are involved in the degradation of arabinoxylans, all of which will affect the structure of polymers known to be important for good dough formation. Their action may influence the flour quality including dough rheology, bread volume, crumb structure and bread storage life. Feruloyl esterases are already added to some dough mixtures depending on the additive supplier to the bakery.

1.2.7.2. *Brewing industry*

The principle structural difference between water-soluble and water-insoluble arabinoxylan from wheat endosperm is that the former contains higher levels of arabinose and lower levels of ferulic acid. The viscosity problems arising from the presence of water-soluble arabinoxylans are undesirable in brewing. Feruloyl esterases could be used to reduce viscosity problems during the filtering of beer. It has been demonstrated that AnFAEA can act as a solubilising agent, releasing β -glucans and pentosans from the starchy endosperm of barley (Moore *et al.*, 1996), suggesting that ferulic acids contribute to the integrity of the endosperm cell walls (Bartolome *et al.*, 1997b). Endogenous feruloyl esterase activity has been detected in stored and germinating grain of barley and wheat (Sancho *et al.*, 1999). The activity of these extracts is comparable to fungal feruloyl esterase in releasing ferulic acid from waste material (Sancho *et al.*, 2001).

1.2.7.3. *Gel formation*

The oxidative cross-linking properties of ferulic acid, which allow the formation of arabinoxylan and pectin gels, are utilised in diverse applications ranging from food ingredients to wound dressing (Ralet *et al.*, 1994a). Figure 1-11 shows how the addition of a small quantity of AnFAEA can affect an oxidatively cross-linked “cold-setting” maize arabinoxylan gel produced by DuPont. The untreated gel was solid, forming a spherical “lump”. With the addition of AnFAEA, an initial hard gel was formed, which lost its rigidity over a few minutes. Understanding and controlling the gel formation via hydrolysis of cross-linking using specific peroxidases and feruloyl esterases could result in a range of arabinoxylan gels with diverse physico-chemical properties.

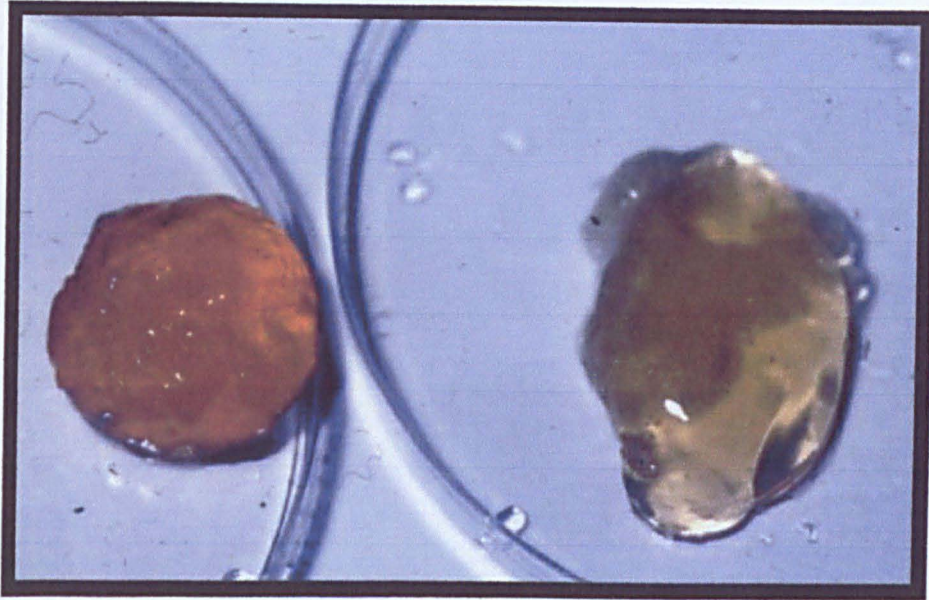


Figure 1-11: Cold-setting arabinoxylan gel. Comparison of a maize arabinoxylan gel without (right) and with (left) the addition of AnFAEA feruloyl esterase from *Aspergillus niger* (Dr. C.B. Faulds, IFR, personal communication).

1.2.7.4. Flavour industry

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component of vanilla flavour extracted from the fermented pods of *Vanilla* orchids. It is the world's principal flavouring compound, used extensively in the food industry. Due to the high cost of the *Vanilla* pod extraction process, and an increasing demand from the food industry, synthetic vanillin is mainly produced from Kraft lignin. Biotechnology offers the possibility to produce a replacement for synthetic vanillin that is produced “naturally” at competitive price (Walton *et al.*, 2000). Microorganisms can be selected for their ability to grow on a putative precursor of vanillin as a sole source

of carbon and energy. This precursor should be available at low cost such as the by-products of the agriculture industry. *Aspergillus niger* has been shown to transform ferulic acid to vanillic acid, which is then bio-converted to vanillin by *Pycnoporus cinnabarinus* or *Pseudomonas fluorescens* (Gasson *et al.*, 1998; Lesage-Meessen *et al.*, 1996; Lesage-Meessen *et al.*, 1999). In order to produce "natural vanillin" it is necessary to use "natural ferulic acid". Release of ferulic acid from agro-industrial waste materials using GRAS (Generally Regarded As Safe) approved enzymes such as feruloyl esterases, would provide a source of "natural vanillin" (Bonnin *et al.*, 2001; Kroon and Williamson, 1999; Williamson *et al.*, 1998b).

1.2.7.5. Health and cosmetic industry

Functional foods are becoming available in many countries and the potential markets are enormous. Functional foods are enriched in biologically active components, which may contribute in some way to improving health. Ferulic acids have effective antioxidant properties with potential applications in the pharmaceutical and food industries. Dietary hydroxycinnamates consist predominantly of ferulic acid and caffeic acid, which protect the body from free radicals and reactive oxygen species (Clifford, 1999; Garcia-Conesa *et al.*, 1999; Kroon and Williamson, 1999; Saija *et al.*, 1999; Williamson *et al.*, 1998b). Ferulic acid derivatives constitute the active ingredient in many skin lotions and sunscreens designed for photoprotection. Cereal brans are considered beneficial for colon health by reducing the risk of colorectal cancers through the nitrite-scavenging properties of ferulic acids. Nitrite is a reactive ion, which can react with secondary amines under acidic conditions to form N-nitroso compounds, many of which are carcinogenic in animals (Jacobs *et al.*, 1995; Moller *et al.*, 1988). Again ferulic acids can be obtained from food processing wastes by feruloyl esterase enzymatic action.

1.2.7.6. Food preservatives

Feruloyl esterases are able to release phenolic compounds, which have industrial applications. Natural and related phenolic compounds have shown antimicrobial properties against bacteria and fungi, and phenolics have long been used as food

preservatives to inhibit microbial growth (Borneman *et al.*, 1986). Phenolic monomeric constituents of lignin have also been implicated as inhibitors of growth and cellulose degradation by ruminal microorganisms (Borneman *et al.*, 1986; Faulds and Williamson, 1999a; Kroon and Williamson, 1999; Williamson *et al.*, 1998b). Because of the growing concerns regarding the safety of synthetic oxidants and food additives, natural antioxidants such as ferulic acids have gained popularity and importance in the last few years. Hydroxycinnamates and their dimeric derivatives have antioxidative potential above standard Vitamin E, and together with their antimicrobial properties could be used as a dual-purpose food antioxidant/antimicrobial preservative (Garcia-Conesa *et al.*, 1999).

1.2.7.7. Farming industry

Wheat, maize and rice brans are low-value waste products of the milling industries. Much of this material is used as animal feed, although the calorific value is low. Pre-treatment with enzymes (xylanases, cellulases) can increase the digestibility and the calorific value. However, these increases are relatively small due to the large quantities of esterified phenolics present in the cell wall materials, which inhibit their digestion by microbial enzymes. The use of feruloyl esterases in pre-treated animal feed to remove esterified phenolics increases the rate of digestion by ruminal microorganisms and relieves toxicity to ruminal bacteria (Williamson *et al.*, 1998b).

Because of the wide range of commercial applications, interest in feruloyl esterases has risen in the food and pharmaceutical industries. Members of this enzyme family have been patented and are currently used in industrial processes.

1.2.8. Microorganisms as biotechnological factories

Microorganisms have proven to be very productive biotechnological factories but there are various drawbacks to the microbial systems currently in use. Many commercially important proteins require correct glycosylation to achieve optimal function. In general, prokaryotic organisms do not facilitate the correct addition of carbohydrate groups during protein synthesis. To overcome this problem eukaryotic

systems have been developed. However, many eukaryotic systems such as animal cells require expensive large-scale growing conditions and many, otherwise suitable eukaryotic systems, may not have the necessary safe status required by regulatory authorities. *Talaromyces emersonii* like *Aspergillus niger*, are eukaryotes, which have “Generally Regarded As Safe” status (GRAS). GRAS is the regulatory status of food ingredients. GRAS status may be either affirmed by the Food and Drug Administration FDA or determined independently by qualified experts. The Food and Drug Administration is part of the Public Health Service of the U.S. Department of Health and Human Services. It is this regulatory agency responsible for ensuring the safety and wholesomeness of all foods sold in interstate commerce except meat, poultry and eggs, which are under the jurisdiction of the U.S. Department of Agriculture (USDA).

In this project, feruloyl esterases have been purified from the two mesophilic fungi *Talaromyces stipitatus* and *Neurospora crassa*, neither of which are human pathogens (Perkins and Davis, 2000). These fungi are part of the Ascomycetes class, from ascus: special type of sporangium enclosing the ascospores, and the sub-class of Euascomycetes, which possess asci enclosed within a fruit body. Fungi are characterised by non-motile bodies (thalli) constructed from apically elongated walled filaments (hyphae), heterotrophic nutrition and life cycle with sexual (teleomorphic stage) and asexual (anamorphic stage) reproduction.

1.2.8.1. *The mesophilic fungus Neurospora crassa*

The filamentous fungus *Neurospora crassa*, is widely used in genetic studies and is the most extensively investigated genus (Radford and Parish, 1997; Schulte *et al.*, 2002). Its genome of 43.0 Mb, dispersed in seven chromosomes, is now almost entirely sequenced and is publicly accessible at www-genome.wi.mit.edu. *Neurospora* species are found in warm, humid climates, with a special preference for colonising decaying vegetation and sites of recent fires, and it is also known as a red bread mould. *N. crassa* has many convenient features as an object of genetic study. It grows rapidly on defined medium, requiring only a common source of carbon such as sucrose and a trace of the vitamin biotin in addition to the usual essential

inorganic salts (Vogel, 1956). *N. crassa* is heterotrophic, requiring a supply of preformed organic compounds for its energy production and growth. It is also known to be saprophytic, living on decaying organic matter and feeding by secreting hydrolytic enzymes, such as cellulases, xylanases, and esterases, into its local environment (Romero *et al.*, 1999; Yazdi *et al.*, 1990a; Yazdi *et al.*, 1990b). These enzymes digest the various polymers to produce soluble products that the fungus can absorb.

1.2.8.2. *The mesophilic fungus Talaromyces stipitatus*

Until May 1984, *Tal. stipitatus* was preserved as *Penicillium stipitatus* based on morphological features; some species of *Penicillium* are the anamorph (asexual) stage of the ascomycete genus *Talaromyces* (Taylor *et al.*, 1990). *Penicillium* is a large group encountered almost everywhere, and usually the most abundant genus of fungi in soils. *Talaromyces stipitatus*, as an heterotrophic and saprophytic organism, is commonly found in soil, dung or decaying plant material and has the ability to secrete hydrolytic enzymes. The thermophilic aerobic fungus *Talaromyces emersonii* has been shown to have high multiplicity in its plant cell wall-degrading enzyme system (Tuohy *et al.*, 1994a; Tuohy *et al.*, 1994b). Thirteen xylanases had been purified from this organism together with the report of feruloyl and *p*-coumaroyl esterase activities. Thus, this genus appeared to be a good candidate to study multiple feruloyl esterase production in order to identify esterases with different characteristics to those already known.

1.3. AIMS OF THIS PHD THESIS

- Identification and cloning of new genes encoding feruloyl esterases from the mesophilic fungi *Neurospora crassa* and *Talaromyces stipitatus*.
- Over-expression of the recombinant feruloyl esterases in a commercially suitable microorganism.
- Purification and characterisation of the recombinant enzymes.
- Identification of amino acid residue(s) involved in the catalytic reaction.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

Media components were purchased from Oxoid (Basingstoke, UK) except for Yeast Nitrogen Base and Sabouraud Dextrose, from Difco (Oxford, UK). Antibiotics and chemicals were purchased from Sigma and BDH (Poole, UK). Oligonucleotide primers were purchased from Sigma Genosys UK. Molecular biology enzymes and other reagents were from purchased from Life Technologies Inc. (Paisley, UK), Promega, Boehringer Mannheim, or Stratagene UK. PCR reactions were performed using deoxynucleoside triphosphate purchased from Promega, *Taq* DNA polymerase purchased from *Abgene* with homemade 10 X PCR buffer 2, *Vent* DNA polymerase with 10 X ThermoPol buffer purchased from BioLabs or *Pfu* DNA polymerase with 10 X Cloned *Pfu* buffer purchased from Stratagene. Long template PCR reactions were carried out using the Expand Long Template PCR System with 10 X buffer 3 purchased from Boehringer Mannheim. The RACE-PCR kit was purchased from Invitrogen Living Science. PCR amplifications were carried out using a Techne Progene thermocycler. The *Escherichia coli* expression system was from Novagen (Nottingham, UK). The *Pichia pastoris* expression system was supplied by Invitrogen Living Science (The Netherlands). The methyl esters of ferulic, caffeic, *p*-coumaric and sinapic acids were obtained from Apin Chemicals, UK. The corresponding free acids and chlorogenic acid and naphthyl acetate were obtained from Sigma-Aldrich Chemical CO (Poole, UK).

2.2. STRAINS AND VECTORS

Escherichia coli TOP10 and TOP 10F' (Invitrogen) were used as the bacterial host for DNA manipulations with the pCR[®]2.1-TOPO[®] and pCR[®]4-TOPO[®] cloning vector (TOPO TA Cloning[®] kit from Invitrogen). *E. coli* GeneHogs[®] (Invitrogen) was used as the host for the *Tal. stipitatus* cDNA library. *E. coli* XL1-Blue MRF' was used as the host for lambda and *E. coli* XL0LR was used as the host in the excision of the pBK-CMV phagemids vector from the lambda ZAP Express vector

(Stratagene #239614). *E. coli* BL21(DE3)pLysS was used to produce recombinant enzymes with the expression vector pET3a (Novagen). *Pichia pastoris* strain GS115 (*his4*) was used to produce recombinant enzymes with the expression vector pPIC3.5K or pPIC9 (Invitrogen). *Talaromyces stipitatus* strain CBS 375.48 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands. The wild type *Neurospora crassa* strain used was the ST A (74 A) St Lawrence No 74-OR23-1A from the Fungal Genetics Stock Center.

2.2.1. *E. coli* strain genotypes

TOP10: F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*.

TOP10F': F' {*lacI*^q Tn10 (Tet^R)} *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*.

GENEHOGS: F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *deoR* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG* *phuA::IS2*.

XL1-Blue MRF': Δ (*mcrA*)183 Δ (*mcrCB-hsdSMR-mrr*)173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* [F' *proAB* *lacI*^q Δ M15 Tn10 (Tet^R)].

XL0LR: Δ (*mcrA*)183 Δ (*mcrCB-hsdSMR-mrr*)173 *endA1* *thi-1* *recA1* *gyrA96* *relA1* *lac* [F' *proAB* *lacI*^q Δ M15 Tn10 (Tet^R)] Su⁻ λ^+ .

BL21(DE3)pLysS: F⁻ *ompT* *hsdS_B* (*r_B* *m_B*) *gal* *dcm* (DE3) pLysS (Cm^R).

2.3. GENERAL METHODS IN MOLECULAR BIOLOGY

2.3.1. Growth and storage of *Escherichia coli* bacteria

From Sambrook *et al.*, 1989.

Bacteria were grown using sterile techniques in either Luria-Bertani Broth (L-broth) (for 1 litre: 10 g NaCl, 10 g tryptone, 15 g yeast extract, pH 7 in the presence of 15 g agar for L-agar) liquid culture medium at 37 °C in a shaking incubator at 250 rpm overnight or on L-agar plates (solid culture medium) in an inverted position at 37 °C overnight, in the presence of the appropriate antibiotic when required. Liquid cultures were inoculated from single colonies from an L-agar plate or grown from

overnight culture diluted 1:100 in L-broth. Long-term storage of bacteria was by addition of 10 % v/v glycerol to aliquots of overnight cultures followed by brief vortex-mixing, flash freezing in liquid nitrogen and storage at - 70 °C. Frozen stock were made for all transformed bacterial strains from which DNA was isolated and analysed by restriction digest or DNA sequencing.

2.3.1.1. *Media additives*

Chloramphenicol: A stock solution of 34 mg ml⁻¹ was made up in 100 % ethanol and stored at - 20 °C. The working concentration was 34 µg ml⁻¹.

Ampicillin: A stock solution of 50 mg ml⁻¹ was made up in water, filter sterilised and stored at - 20 °C. The working concentration was 50 µg ml⁻¹.

Tetracycline: A stock solution of 12.5 mg ml⁻¹ was made up in 100 % ethanol and stored at - 20 °C. The working concentration was 12.5 µg ml⁻¹.

Kanamycin: A stock solution of 50 mg ml⁻¹ was made up in water, filter sterilised and stored at - 20 °C. The working concentration was 50 µg ml⁻¹.

IPTG: A stock solution of 100 mM was made up in water, filter sterilised and stored at - 20 °C. For pCR[®]2.1-TOPO[®] vector we used 10 µl of the stock solution per agar plate.

X-gal: A stock solution of 2 % (w/v) was made up in N,N' dimethylformamide and stored at - 20 °C. For pCR[®]2.1-TOPO[®] vector we used 80 µl of the stock solution per agar plate.

2.3.2. *Fungal growth conditions*

2.3.2.1. *Growth conditions for Talaromyces stipitatus*

Tal. stipitatus strain CBS 375.48 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands and maintained on Sabouraud Dextrose agar (Difco; 1.5 % w/v) as 5-day old slopes (Moloney *et al.*, 1983). A 1cm² piece of mycelial mat was incubated in 100 ml of Sabouraud-dextrose (SD) medium enhanced with 1 ml of 10 % (w/v) glucose at 25 °C under agitation at 2500 rpm. The culture was vacuum filtered through a sterile Buchner funnel and washed with sterile

distilled water, before aseptic transfer to inducing media of either sugar beet pulp (SBP) or wheat bran (WB) and incubated at 25 °C with shaking for several days. The medium was filtered, the fungal mat was discarded and the filtrate, which contains fine particles of SBP or WB, autoclaved. Meanwhile mycelial mats were grown in SD medium supplemented with glucose as before. The culture was filtered and washed, then transferred to the pre-digested media and incubated for 6 or 8 hours. The resulting mycelial growth was harvested by filtration, washed thoroughly with sterile distilled water and RNAs extracted as rapidly as possible.

1L of selective culture media contained: 2 % (w/v) carbon source, 0.5 % (w/v) corn steep liquor, 114 mM ammonium sulphate, 36 mM KH_2PO_4 , 0.1% (w/v) yeast extract and 0.1 % (v/v) salt solution.

Salt solution contains 2.5mg of the following salts per litre of growth media: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot \text{H}_2\text{O}$, KI, Na_2MoO_4 , boric acid.

After titration to pH 4.5 and autoclaving, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 5g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5g/l, $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ 10g/l, were added aseptically.

2.3.2.2. Growth conditions for *Neurospora crassa*

Neurospora crassa wild type ST A (St Lawrence 74-OR23-1A) was maintained on minimal Vogel's agar (1.5 % w/v) plus 2 % sucrose (Vogel, 1956). A loop full of conidia grown slope culture was grown in 100 ml of Vogel's sucrose medium at 30 °C, shaking at 200 rpm. The culture was vacuum filtered through a sterile Buchner funnel, washed with sterile distilled water, aseptically transferred to the pre-digested inducing media and incubated for 6 hours. Vogel's SBP or WB media were pre-digested by *N. crassa* and autoclaved following the protocol described above. The mycelia were then harvested by filtration and RNA extraction performed.

1L Vogel's 50 X concentrate: Na_3 citrate 5.5 g, H_2O 150 g, KH_2PO_4 250 g, NH_4NO_3 100g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 10 g, CaCl_2 5 g, Trace elements 5 ml, 0.25 mg D-biotin, and a few drops of chloroform as preservative.

100 ml trace elements: citric acid H_2O 5 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 5g, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ 1 g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.25 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.05 g, H_3BO_3 0.05 g, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.05 g, and a few drops of chloroform as preservative.

Vogel's minimum medium is supplemented by 2 % (w/v) carbon source (sucrose, sugar beet pulp or wheat bran).

2.3.3. Yeast growth conditions

Pichia pastoris GS115 (*his4*), obtained from Invitrogen, was grown at 30 °C in YPD medium or BMGY medium and transformants were recovered on regeneration dextrose (RD) plates. Minimal Dextrose (MD) and Minimal Methanol (MM) plates were used to assess the Methanol Utilisation phenotype of *Pichia* transformants. For the expression of recombinant enzymes, BMMY medium was used.

2.3.4. Yeast media

2.3.4.1. Stock solutions

10 X D (20 % (w/v) Dextrose)

20 g D-glucose was dissolved in 100 ml of water, sterilised by autoclaving and stored at room temperature.

10 X YNB (13.4 % (w/v) Yeast Nitrogen Base)

13.4 g of yeast nitrogen base with ammonium sulfate but without amino acids was dissolved in 100 ml of water, filter sterilised and stored at 4 °C.

500 X B (0.02 % (w/v) Biotin)

20 mg biotin was dissolved in 100 ml of water, filter sterilised and stored at 4 °C.

100 X H (0.4 % (w/v) Histidine)

400 mg of L-histidine was dissolved in 100 ml of water, filter sterilised and stored at 4 °C.

10 X AA (0.5 % (w/v) of each Amino Acids)

500 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine, L-isoleucine was dissolved in 100 ml of water, filter sterilised and stored at 4 °C.

10 X GY (10 % (v/v) Glycerol)

100 ml of glycerol c with 900 ml of water, sterilised either by filtering or autoclaving and stored at room temperature.

10 X M (5 % (v/v) Methanol)

5 ml of methanol was mixed with 95 ml of water, filter sterilised and stored at 4 °C.

1 M potassium phosphate buffer, pH 6.0

132 ml of 1M K₂HPO₄ and 868 ml of 1M KH₂PO₄ were mixed together, sterilised by autoclaving and stored at room temperature.

2.3.4.2. Media*YPD or YEPD (Yeast Extract Peptone dextrose Medium) (1 litre)*

10 g yeast extract, 20 g peptone, 20 g D-glucose, 20 g agar for plates.

The yeast extract and peptone were dissolved in 900 ml of water, autoclaved before adding 100 ml of 10 X D and stored at 4 °C.

RDB and RDBH (Regeneration Dextrose Base ± Histidine) plates (1 litre)

186 g sorbitol, 20 g agar, 20 g D-glucose, 13.4 g yeast nitrogen base, 0.4 mg biotin, 50 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine.

186 g sorbitol and 20 g agar were mixed in 700 ml of water and autoclaved, then cooled to 45 °C. To this was added a pre-warmed mix of 100 ml 10 X D, 100 ml 10 X YNB, 2 ml 500 X B, 10 ml 100 X AA, ± 10 ml 100 X H made up to 300 ml with water. Plates were stored at 4 °C.

MD (Minimal Dextrose) plates (1 litre)

15 g agar, 20 g D-glucose, 13.4 g yeast nitrogen base, 0.4 mg biotin.

15 g agar were mixed with 800 ml of water and autoclaved then cooled to 55 °C. To this was added a pre-warmed mix of 100 ml 10 X YNB, 2 ml 500 X B and 100 ml 10 X D. Plates were stored at 4 °C.

MM (Minimal Methanol) plates (1 litre)

15 g agar, 13.4 g yeast nitrogen base, 0.4 mg biotin, 5 ml methanol.

Prepared as for MD plates, with 10 X M in place of 10 X D.

BMGY (Buffered complex Glycerol Medium) (1 litre)

10 g yeast extract, 20 g peptone, 0.1 M potassium phosphate buffer pH 6, 13.4 g yeast nitrogen base, 0.4 mg biotin, 100 ml glycerol.

10 g yeast extract and 20 g peptone were dissolved in 700 ml of water and autoclave, then cooled to room temperature before adding 100 ml 1 M potassium phosphate buffer pH 6, 100 ml 10 X YNB, 2 ml 500 X B and 100 ml 10 X GY. The buffer was stored at 4 °C before use.

BMMY (Buffered complex Methanol Medium) (1 litre)

10 g yeast extract, 20 g peptone, 0.1 M potassium phosphate buffer pH 6, 13.4 g yeast nitrogen base, 0.4 mg biotin, 5 ml methanol.

Prepare as for BMGY, adding 10 X M in place of 10 X GY and stored at 4 °C.

2.3.5. Extraction of fungal genomic DNA

Genomic DNA was extracted from *Tal. stipitatus* and *N. crassa* using the method of Stevens and Metzenberg (Stevens and Metzenberg, 1982). Mycelial mats were incubated for 5 days in 100 ml of Sabouraud-dextrose medium supplemented with glucose or Vogel's minimum medium, respectively at 25 and 30 °C. Cultures were harvested by vacuum filtration, washed thoroughly with 0.9 % (w/v) NaCl, frozen in liquid nitrogen and freeze dried overnight. The dry mats were then pulverised using a glass rod. Two millilitres of extraction buffer (250 mM triethylammonium-EDTA pH 8.1, 50 mM NaCl, 0.5 % (w/v) Triton X-100, 0.25 µg/ml pronase) were added per 50 mg of powder and the suspension gently shaken for 1 – 2 days at 37 °C. Cell residue was pelleted by centrifugation at 8 000 g for 15 minutes and discarded. The supernatant was precipitated with two volumes of 100 % ethanol at room temperature then incubated for 15 minutes and centrifuged as before. The pellet was resuspended in 500 µl of low salt buffer (1 mM NaCl, 1 mM Tris-HCl pH 7.4, 0.1 mM Na₂-EDTA). To this were added 3 ml of ethanolic perchlorate reagent (36 ml

distilled water added to 140 g of $\text{NaClO}_4 \cdot \text{H}_2\text{O}$, heated to 65 °C and carefully added to 381 ml 100 % ethanol) and mixed by inversion. The DNA was pelleted by centrifugation at 12 000 g for 3 minutes. The DNA was drained and re-dissolved in 500 µl low salt buffer, 250 µl high salt buffer (100mM NaCl, 25mM Tris-HCl pH 7.4, 2mM EDTA) and 300 µg/ml preboiled RNase A. The preparation was then incubated for 3 hours at 37 °C, mixed with an equal volume chloroform:isoamyl alcohol (24:1 v/v) and mixed for 2 - 24 hours on a “windmill” rotator. The phases were separated by centrifugation at 12 000 g for 5 minutes. The upper phase was removed and transferred to a fresh tube containing 450 µl isopropanol then mixed by inversion and left at – 70 °C for 30 minutes to precipitate the DNA. The DNA was recovered by centrifugation as above, resuspended in 200 µl low salt buffer and re-precipitated with 600 µl 100 % ethanol for 30 minutes at room temperature. The DNA pellet was resuspended in sterile distilled water and stored at 4 °C.

2.3.6. Alkaline lysis “mini-prep” method for preparation of plasmid DNA

A single colony was picked from an L-agar plate containing appropriate antibiotics to maintain selection of the plasmid and cultured overnight in 10 ml L-broth also containing antibiotic for selection. Approximately 1.5 – 3 ml of the overnight culture was pelleted by centrifugation at 12 000 g for one minute in an eppendorf tube and the supernatant discarded. The pellet was resuspended in 100 µl of Solution I and incubated at room temperature for 5 minutes. To this, 200 µl Solution II was added, mixed gently, and incubated on ice for 5 minutes followed by addition of 150 µl Solution III and incubated on ice for a further 15 minutes. After centrifugation at 12 000 g for 15 minutes at 4 °C, the supernatant was transferred to a fresh eppendorf tube and the DNA precipitated with 1 ml 100 % cold (– 20 °C) ethanol. After centrifugation at 12 000 g for 10 minutes at 4 °C, the pellet was resuspended in 300 µl sterile distillate water followed by addition of 1 U RNase-A plus 150 µl 7.5 M ammonium acetate and incubated at – 20 °C for 15 minutes. After centrifugation at 12 000 g for 10 minutes at 4 °C, the supernatant was transferred in a fresh eppendorf tube and the DNA precipitated with 0.1 volumes 3 M sodium acetate pH 5.2 and 2.5

volumes 100 % cold (- 20 °C) ethanol. After centrifugation at 12 000 g for 15 minutes at 4 °C, the pellet was washed by addition of 500 µl 70 % (v/v) ethanol and centrifugation at 12 000 g for 5 minutes at room temperature. The resulting pellet was resuspended in 30 - 60 µl sterile distilled water and analysed by restriction digest or DNA sequencing.

Solution I: 50 mM glucose, 25 mM Tris-HCl pH 7.8, 10 mM EDTA pH 7.8. Sterilise by autoclaving.

Solution II: 0.2 N NaOH, 1 % SDS.

Solution III: 60 ml KOAc 5 M, 11.5 ml glacial acetic acid, 28.5 ml H₂O.

2.3.7. Fungal RNA extraction

RNA is subject to rapid degradation, therefore only freshly autoclaved tips, Eppendorf tubes and sterile distilled water were used.

RNA was extracted using the method described by Sokolovsky (Sokolovsky *et al.*, 1990). Mycelial mats were harvested by filtration, thoroughly washed with sterile distilled water and ground to a powder under liquid nitrogen. The powder was transferred straight away into microcentrifuge tubes containing equal volumes (0.65 ml) of lysis buffer (0.6 M NaCl, 10 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 4 % (w/v) SDS), and phenol (saturated with 0.1 M Tris-HCl pH 8.0). The tubes were mixed on a windmill rotator (or a shaking tray) for 20 - 30 minutes and centrifuged at 12 000 g for 10 minutes. Supernatants were transferred to microcentrifuge tubes containing equal volumes of phenol (pH 8.0) and vortex mixed for 20 seconds. The phases were separated by centrifugation (12 000 g for 10 minutes) and the upper phases transferred to equal volumes of 8 M LiCl. After overnight storage at 4 °C the tubes were vortex mixed for 20 seconds and centrifuged as before. The pellets were resuspended in 0.3 ml sterile distilled water and precipitated by addition of 30 µl 3 M sodium acetate pH 5.2 and 0.75 ml ethanol, mixed by inversion and incubated at - 70 °C for 1 hour. The RNA was pelleted by centrifugation at 12 000 g for 10 minutes. The pellets were washed by addition of 500 µl 70 % (v/v) ethanol, centrifuged at 12 000 g for 10 minutes, dried at room temperature for 2 minutes and resuspended in 50

- 100 µl sterile distilled water. The RNA was analysed by electrophoresis on 0.8 % (w/v) agarose gels. RNA was stored at - 70 °C in 0.1 volumes 3 M sodium acetate pH 5.2 and 2.5 volumes 100 % ethanol.

Total RNAs were extracted in duplicate experiments from mycelia following growth on pre-digested media (minimal Vogel's supplemented with 2 % (w/v) SBP or WB) and Vogel's medium with 2 % (w/v) sucrose (*N. crassa*) or Sabouraud-dextrose (*Tal. stipitatus*). In addition, RNAs were extracted every 48 hours in independent duplicate experiments during the fermentation of SBP and WB by *N. crassa*.

2.3.8. DNase treatment of RNA

To remove contaminating genomic DNA, RNA samples were treated with RNase-free DNaseI supplied by Promega (RQ1 RNase-Free DNase). One unit of enzyme (1 U/µl) used to treat 1 µg of RNA. The digestion was performed according to the manufacturer's procedure, at 37 °C for 30 minutes. Phenol:chloroform (v/v) extraction and ethanol precipitation were performed after the digestion. The RNA integrity was checked by agarose gel electrophoresis (integrity of the rRNA bands) before using the RNA in RT-PCR (Reverse Transcription-Polymerase Chain Reaction) or RACE-PCR (Rapid Amplification of cDNA-ends-Polymerase Chain Reaction) reactions.

2.3.9. RNA separation in formaldehyde agarose gel

In all cases the gel tank, former and comb were treated with 0.1 M NaOH, 1 % SDS for 30 minutes before rinsing with freshly autoclaved sterile distilled water. Gloves, eyes and mouth protection were employed and the experiments were carried out in a fume hood.

Agarose (1.2 % (w/v)) formaldehyde (6.6 % (v/v)) gels were prepared as follows: 0.6 g of agarose was melted in 36 ml of distilled water and allowed to cool to approximately 55 °C and mixed with 5 ml of 10 X running buffer (0.2 M MOPS, 10 mM EDTA, 10 mM sodium acetate, pH 7.0) and 9 ml of 37 % (v/v) formaldehyde.

The gel was cast and allowed to solidify for 1 hour at room temperature in a fume hood. Total RNA (~5 µg) was precipitated with 1/10 volume 3 M sodium acetate pH 5.2 and 2 volumes of ethanol. The pellet was resuspended in 2 µl of sterile distilled water. Formamide (5 µl), formaldehyde (2 µl), 10 X running buffer (1 µl), and 400 µg/ml ethidium bromide (1 µl) were added to each sample. The samples were then mixed thoroughly and heated for 10 minutes at 65 °C. After heating, the samples were placed on ice. Following a brief centrifugation to collect any condensate, 1 µl of 10 X loading buffer (0.2 % (w/v) bromophenol blue, 10 mM EDTA, 50 % (w/v) glycerol) was added. The gel was electrophoresed overnight at 1.5 V/cm in 1 X running buffer.

2.3.10. Spectrophotometric determination of nucleic acid concentration and purity

Both DNA and RNA absorb at wavelengths of 260 and 280 nm and their concentration can be calculated by measuring absorbance at 260 nm (Sambrook *et al.*, 1989). An OD₂₆₀ of 1 corresponds to a concentration of 50 µg ml⁻¹ for double stranded DNA, 40 µg ml⁻¹ for RNA, and 33 µg ml⁻¹ for single stranded DNA. The ratio of absorbance at 260 and 280 nm (OD₂₆₀/OD₂₈₀) gives a guide to the purity of the sample. Pure preparations of DNA and RNA have OD₂₆₀/OD₂₈₀ values of 1.8 and 2.0 respectively. If there is contamination with protein or phenol this ratio will be less and accurate quantification is not possible.

2.3.11. Restriction digestion of DNA

Digestion of DNA by restriction enzymes was carried out using the conditions recommended by the enzyme supplier. Typically, digestions were performed at the appropriate temperature for 2 hours in a total volume of 10 - 50 µl with approximately 0.5 - 2 units of enzyme per 0.2 - 1.5 µg of DNA using the appropriate restriction buffer supplied with the enzyme. If the DNA preparation to be digested contained RNA, RNase (1 U/µg DNA) was also included in the digestion reaction.

2.3.12. Agarose gel electrophoresis

From Sambrook *et al.*, 1989.

A suspension of agarose (typically 0.8 % (w/v)) in TAE buffer (40 mM Tris-Acetate, 2 mM EDTA, pH 7.9) was melted using a microwave oven. Ethidium bromide was added to a concentration of $1 \mu\text{g ml}^{-1}$. The agarose was poured into a casting tray and allowed to set. Once set, the gel was immersed in a buffer tank containing TAE buffer. Samples to be loaded were mixed with 0.2 volumes loading buffer (50 % (v/v) glycerol, 0.025 M EDTA pH 8.0, 0.01 % (w/v) bromophenol blue powder). Samples were loaded and a constant voltage (45 - 150 V) applied across the gel. At neutral pH nucleic acids have a negative charge and so migrate towards the anode. The DNA loading buffer contains the bromophenol blue dye so the migration of the DNA could be monitored in the electric field. Separation by agarose gel electrophoresis could be used to size DNA fragments by comparing their migration with that of fragments of known sizes. The ethidium bromide in the gel intercalates with nucleic acids causing it to fluoresce under UV irradiation so that band positions could be visualised.

2.3.13. Ligation reaction

T4 DNA ligase (Stratagene, UK) was used to join DNA strands between 5'-phosphate and 3'-hydroxyl groups of adjacent nucleotides with cohesive or blunt ends. Generally a molar ratio of insert to vector of 3:1 was used when cloning a fragment into a plasmid vector. Added to the vector and insert DNAs (total DNA 0.01 - 1 μg) were 0.5 μl of T4 DNA ligase (4 U/ μl), 1 μl of 10 mM ATP, 1 μl of 10 X ligase buffer, and water to a final volume of 10 μl . The reaction was incubated at either room temperature for 3 hours or 4 °C overnight or 15 °C for 4 - 18 hours. The optimal temperature for the ligation reaction is a balance between the optimal temperature for T4 DNA ligase enzyme activity (25 °C) and the temperature necessary to ensure annealing of fragment ends.

2.3.14. Preparation of chemically competent *E. coli* cells

A single colony of the *E. coli* host strain of choice was picked from an L-agar plate and used to inoculate 10 ml L-broth containing the required antibiotic (tetracycline for TOP10F' cells and chloramphenicol for BL21(DE3)pLysS cells) and grown at 37 °C overnight. The overnight culture (1 ml) was used to inoculate 4 x 100 ml of L-broth containing the appropriate antibiotic, in 4 x 1 Litre flasks. The cells were grown at 37 °C with vigorous shaking to an OD₅₅₀ of 0.45 - 0.55. The cells were cooled on ice for 15 minutes and pelleted by centrifugation at 2 500 g at 4 °C for 10 minutes. The supernatant was discarded and the pellet gently resuspended in 120 ml of ice-cold RF1 Buffer. After incubation on ice for 15 minutes the cells were centrifuged as before and the pellet resuspended in 30 ml of ice-cold RF2 Buffer. After incubation on ice for 15 minutes the cells were aliquoted (200 µl) into pre-chilled eppendorf tubes, flash frozen in liquid nitrogen and then stored at - 70 °C.

RF1 Buffer pH 5.8 (500 ml): 6 g RbCl, 4.95 g MnCl₂, 0.75 g CaCl₂, 75 ml 1 M KOAc. Filter sterilised.

RF2 Buffer pH 6.8 (500 ml): 0.6 g RbCl, 10 ml of 0.5 M MOPS pH 6.8, 5.5 g CaCl₂, 75 ml glycerol. Filter sterilised.

2.3.15. Transformation of chemically competent *E. coli* cells

Competent cells were thawed on ice. Plasmid DNA (10 - 100 ng) or ligation reactions in a maximum volume of 10 µl were added to 200 µl aliquots of the thawed competent cells and incubated on ice for 45 minutes. The cells were heat shocked for 45 seconds at 42 °C, incubated on ice for 2 minutes, followed by the addition of 0.5 ml L-broth (containing no antibiotic). The cells were incubated at 37 °C for one hour with gentle agitation to initiate expression and replication of the plasmid to ensure expression of the antibiotic resistance gene(s). Aliquots of 50 - 100 µl of the cell suspensions were spread onto L-agar plates (containing the required antibiotics) and incubated overnight at 37 °C. To assess insertional activation of the α-peptide of β-galactosidase, plates containing 80 µl X-gal 2 % (w/v) and 10 µl IPTG 100 mM were employed.

2.3.16. Screening for insertional activation (Blue/white screening)

To produce an enzymatically active β -galactosidase in the cloning host requires that an interaction occurs between two protein domains: the α -peptide coded by a plasmid vector and a truncated form of the enzyme encoded on the bacterial chromosome (the Δ M15 deletion mutation of the β -galactosidase gene; *lacZ*). These two domains interact to form a functional enzyme, the α -region complementing the missing amino acids resulting from the Δ M15 mutation. IPTG, a chemical analogue of lactose, is necessary for specific induction of *lacZ* transcription. When functional β -galactosidase is produced it converts colourless X-gal into a blue product. The multiple cloning sites (MCS) of plasmid vectors are flanked by DNA coding for the N-terminal region of β -galactosidase (α -peptide). Therefore when additional DNAs are inserted into the polylinker, then expression from the *lacZ* gene is disrupted and white colonies are generated. Non-recombinants colonies remain blue in the presence of X-gal due to the expression of β -galactosidase.

2.3.17. TOPO TA Cloning® of PCR amplified DNA products

PCR amplified DNA products were cloned into pCR®-TOPO® vectors using the TOPO TA Cloning kit from Invitrogen for the TA cloning of *Taq* polymerase-generated PCR products. *Taq* polymerase has a non template-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The pCR®-TOPO® vectors are supplied linearised with 3'-deoxythymidine (T) residue overhangs allowing PCR inserts to ligate efficiently with the vector. Moreover, the vector is covalently bound to the enzyme Topoisomerase I from *Vaccinia* virus, which exploits the ligation reaction of the topoisomerase enzyme by providing and "activated" linearised TA vector. The ligation occurs within 5 minutes at room temperature. The PCR product is mixed with the pCR®-TOPO® vector as described in the kit protocol, incubated at room temperature for 5 minutes then placed on ice. A 2 μ l aliquot of the reaction was used to transform TOP10 or TOP10F' *E. coli* competent cells, which were then plated onto L-agar plates containing ampicillin and X-gal as well as IPTG when using TOP10F' *E. coli*

cells. Screening for insertional activation of the α -peptide of β -galactosidase was only used with the pCR[®]2.1-TOPO[®] vector. The pCR[®]4-TOPO[®] vector allows direct selection of recombinants via disruption of the lethal *E. coli* gene *ccdB* (Bernard *et al.*, 1994). Therefore, X-gal and IPTG were not required when using the pCR[®]4-TOPO[®] vector.

2.3.18. Northern blots

RNAs were electrophoresed in formaldehyde denaturing agarose gels with 1 X running buffer and blotted by capillary transfer with 20 X SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) onto Hybond-N⁺ nylon membrane (Amersham Pharmacia biotech), at room temperature overnight. RNAs were UV cross-linked to the membrane and probed in DIG Easy Hyb formamide based buffer (Boehringer Mannheim) at 42 °C with PCR fragments specific to the *fae-1* or *faeC* genes. These were labelled using the DIG-labelling system (Boehringer Mannheim). Digoxigenin-11-dUTP (DIG-dUTP) can be incorporated by Taq DNA Polymerase during the polymerase chain reaction. The resulting probes are sensitive as the yield from the labelling reaction is high. Colorimetric detection was performed in the presence of NBT (45 μ l of 75 mg ml⁻¹ Nitroblue tetrazolium salt in 70 % (v/v) dimethylformamide) and BCIP (35 μ l of 50 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100 % dimethylformamide), substrates of the alkaline phosphatase enzyme conjugated to the antibodies anti-digoxigenin.

2.3.19. Southern blots

Genomic DNAs were digested with the restriction enzymes and electrophoresed in agarose gels with 1 X TAE running buffer (40 mM Tris Acetate, 2 mM EDTA, pH 7.9). The gels were blotted by capillary transfer with 20 X SSC buffer onto Hybond-N⁺ nylon membranes, at room temperature overnight. DNAs were UV cross-linked to the membrane and probed in DIG Easy Hyb formamide based buffer (Boehringer Mannheim) at 42 °C with PCR fragments labelled using the DIG-labelling system (Boehringer Mannheim). The colorimetric detection was performed in the presence of NBT and BCIP substrates as above.

2.3.20. cDNA library screen

As a project decision a cDNA library was commissioned from a commercial source. To achieve this RNA was supplied from *Tal. stipitatus* to Research Genetics (USA). RNAs were extracted from *Tal. stipitatus* following 6 hours of growth with sugar beet pulp in order to produce a cDNA library.

2.3.20.1. Characteristics of the cDNA library

Table 2-1: Characteristics of the *Tal. stipitatus* cDNA library

DNA concentration	Quantity	Cloning vector	Cloning sites	Expected cfu	Fragment size range
0.6 µg/µl	53 µg (pure cDNAs)	pT7T3D-Pac (modified)	<i>EcoRI</i> , <i>NotI</i>	> 10 ⁶ cfu	1.5 to 2.0 kbp

The cDNA library (Table 2-1) was constructed by reverse transcription of oligo (dT)-primed RNA and directionally cloned (Soares, 1994). The library was passed through a single round of amplification and supplied for direct electroporation into GeneHogs[®] Electrocompetent cells.

2.3.20.2. Transformation of electrocompetent competent *E. coli* cells

50 µl of GENEHOGS[®] Electrocompetent cells were transformed with 1 µl of the cDNAs library (0.6 µg) according to the manufacturer's procedure (Invitrogen #25-0387). The DNA was added to the cells and transferred into a pre-chilled 0.2 cm sterile electroporation cuvette on ice. The outside of the cuvette was dried and placed in the electroporator. Electroporation was performed at 100 Ω, 2.5 kV and 25 µF, followed by a quick addition of 250 µl room temperature L-broth medium. The cells were transferred into a 15 ml screw-cap tube and shaken for at least one hour at 37 °C to allow expression of the antibiotic resistance gene. The transformation reaction was diluted 100-fold and 20 - 150 µl were spread on L-agar plates (containing

ampicillin antibiotic) and incubated overnight at 37 °C.

2.3.20.3. Plating and screening

Around 6,000 colonies were plated (~600 colonies/plate). Colony lift was performed as followed: Hybond-N⁺ nylon circular membranes were placed onto the surface of the agar plates and left for 1 minute. The membranes were carefully removed and blotted briefly on dried Whatman[®] 3MM paper. The membranes were then placed for 15 minutes on filter paper soaked with the denaturation solution (0.5 N NaOH, 1.5 M NaCl, 0.1 % (w/v) SDS) followed by 15 minutes on filter paper soaked with the neutralisation solution 2 (1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl) and 10 minutes on filter paper soaked with the 2 X SSC (20 X SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0). DNAs were UV cross-linked to the membrane and probed using DIG-labelled *faeC* from *Tal. stipitatus* under high stringency conditions (42 °C in DIG Easy Hyb formamide based hybridisation buffer) and washing steps (55 - 65 °C). The colorimetric detection was performed in the presence of NBT and BCIP substrates as above.

2.3.21. Construction of genomic libraries

The lambda ZAP Express[®] Predigested Vector Kit and ZAP Express[®] Predigested Gigapack[®] Cloning Kits (*Bam*HI/CIAIP-Treated) were purchased from Stratagene and used for the synthesis of a genomic library from *Tal. stipitatus*.

2.3.21.1. Partial digestion

A total of 8 µg of genomic DNA was partially digested with *Sau*3AI restriction enzyme, which releases cohesive ends compatible with those of *Bam*HI. Four reactions were performed with different enzyme concentration. For each reaction 2 µg of genomic DNA was incubated with either 0.1 unit of enzyme/µg of DNA, 0.05 U/µg, 0.025 U/µg or 0.0125 U/µg at 37 °C for 25 min. Once partially digested the DNA was loaded onto a low melting point agarose gel (0.6 % (w/v) agarose) and run for 10 - 15 min at 50 V. DNA sized above 600 bp was excised and gel-purified

before ligation into the lambda ZAP Express[®] vector and packaged according to the manufacturer's procedure (Stratagene # 239615).

2.3.21.2. *Plating*

After the packaging reaction the library titer was determined as 1×10^5 pfu/ml. The total packaging reaction (500 μ l) was plated (~3,000 plaques/plate) and plaque lifted for subsequent hybridisation screening.

2.3.21.3. *Plaque lifts, hybridisation and screening*

Plaque lifting, hybridisation and screening were performed according to the manufacturer's procedure (Boehringer Mannheim) using the DIG system (non-radioactive probes). The plaque lift procedure was performed as described above for the colony lift with the membranes placed for 5 minutes on filter paper soaked with the denaturation solution 1 (0.5 N NaOH, 1.5 M NaCl). High stringency conditions were employed for the hybridisation (42 °C in DIG Easy Hyb formamide based hybridisation buffer) and washing steps (55 - 65 °C) when using the homologous probe encoding FAEC. The colorimetric detection was performed in the presence of NBT and BCIP substrates as above.

2.3.21.4. *Single clone excision*

Plaques of interest were cored from the agar plate and the pBK-CMV double-stranded phagemid vector excised following the manufacturer's protocol. Vector DNA was prepared and used either for Southern blot or directly submitted for DNA sequencing.

2.3.22. **Construction of size-restricted genomic libraries**

The lambda ZAP Express[®] Predigested Vector Kit and ZAP Express[®] Predigested Gigapack[®] Cloning Kit (*Eco*RI/CIAP-Treated) were purchased from Stratagene and used for the synthesis of the size restricted genomic library from *Tal. stipitatus*.

Three μ g of genomic DNA was digested overnight with *Eco*RI. The digested fragments were loaded onto a 0.8 % (w/v) agarose gel and run for 2 hours at 55 V. DNA fragments sized between 2.5 and 3.5 kbp and between 5.5 and 7.0 kbp were excised and gel-purified before ligation into the lambda ZAP Express® vector and packaged according to the manufacturer's procedure (Stratagene # 239615). Plating the library, plaque lifts, hybridisation, screening and single-clone excision were performed as described above.

2.4. CLONING OF FERULOYL ESTERASE CODING REGIONS

2.4.1. Cloning of *Neurospora crassa* *fae-1* cDNA by overlapping-PCR

Comparative sequence analysis of the *fae-1* genomic clone, issued from a *Neurospora crassa* genomic library screen, indicated a reading frame of 876 bp was present but interrupted by a single intron, which need to be removed before protein expression can be attempted. Overlapping-PCR amplification was used in order to amplify a contiguous coding region for the *N. crassa* ferulic acid esterase gene (*fae-1*) from genomic DNA.

Two specific primers were designed on either side of a putative intron (Nc-ssN1F, Nc-I1R primers 5' of the intron and Nc-I1F, Nc-C1R primers 3' of the intron shown in Table 2-2). The reverse primer (Nc-I1R) has a tail, the sequence of which complements the forward primer (Nc-I1F) and vice versa. Two PCR reactions were performed to amplify separately the sequences upstream and downstream of the intron using the primer combinations Nc-ssN1F/Nc-I1R and Nc-I1F/Nc-C1R. DNA amplification was carried out through 30 cycles of denaturation (30 sec at 94 °C), annealing (1 min at 60 °C), and extension (1 min at 72 °C) followed by a further 10 min extension at 72 °C, using 50 pmol of each of the forward and reverse primers, 500 mM of each deoxynucleoside triphosphate, 0.1 volume of 10 X PCR buffer 2 (500 mM Tris-HCl pH 9.2, 140 mM (NH₄)₂SO₄, 22.5 mM MgCl₂) and 2.5 U *Taq* DNA polymerase in a reaction volume of 50 μ l. Amplified fragments were gel-purified and an equal quantity of each mixed together. The DNA mix was then denaturated at 100 °C for 10 min and the reaction was allowed to cool slowly at

room temperature. Deoxynucleoside triphosphate (0.5 μ l, 25 mM), 10 X Klenow buffer (2 μ l), Klenow enzyme (1 μ l, 2 U/ μ l) (Boehringer Mannheim) and H₂O were added to make the reaction to a final volume of 20 μ l. The reaction mix was incubated at 37 °C overnight to allow synthesis of double stranded DNA. DNA was precipitated with 0.1 volumes of 3M sodium acetate pH 5.2 and 2.5 volumes of 100 % ethanol. The DNA was dissolved in water before cloning. Alternative *Fae-1* cDNAs with or without the native signal sequence were similarly PCR amplified with primers designed on 5' and 3'-ends of the *fae-1* cDNA (Nc-ssN1F or Nc-N1F, respectively and Nc-C1R, Table 2-2). DNA amplification was carried out through 30 cycles of denaturation (30 sec at 94 °C), annealing (1 min at 60 °C), and extension (1 min at 72 °C) followed by 72 °C for 10 min. The resulting PCR product was cloned into the pCR[®]2.1-TOPO[®] vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

Table 2-2: Oligonucleotide sequences used to amplify the *N. crassa fae-1* cDNA.

Primer names	Primer sequences with their translations*
Nc-ssN1F	5'-AAAAGGATCCCATATG TTG CCC AGA ACA TTG C-3' M L P R T L L
Nc-N1F	5'-AAAAGGATCCCAT ATG GCC TCG CTG CAG CAG GTG-3' M A S L Q Q V
Nc-C1R	5'-AAAAGGATCCGCGGCCG CGA CAT CTA GTT GAT CAA CCC-3' † N I L G
Nc-I1F	5'-C GCC ATT ATT GTG GCT CTC CAC GGC TGT GGC GG-3' A I I V A L H G C G
Nc-I1R	5'-GCC ACA GCC GTG GAG AGC CAC AAT AAT GGC GGG C-3' G C G H L A V I I A P

*Restriction sites incorporated into the primer sequences are underlined (*Bam*HI: GGATCC; *Not*I: GC GGC CGC; *Nde*I: CATATG). †Indicates the stop codon.

2.4.2. Cloning of *Talaromyces stipitatus* *faeC* gene and cDNA

2.4.2.1. Polymerase chain reaction

PCR reactions were performed with genomic DNA (10 ng) as template in 50 µl reaction mixtures containing 50 pmol of each of the forward and reverse primers, 500 mM of each deoxynucleoside triphosphate, 0.1 volume of 10X PCR buffer 2 and 2.5 U *Taq* DNA polymerase. PCR amplifications were carried out by using 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 1 min primer extension at 72 °C for 35 cycles followed by 72 °C for 10 min. Degenerate primers were designed after reverse translation of the N-terminal and internal protein sequences. PCR reactions were performed using the primer combinations TsFAEC-N1F/TsFAEC-I3R, TsFAEC-I1F/TsFAEC-I3R, TsFAEC-I2F/TsFAEC-I3R, TsFAEC-I4F/TsFAEC-I3R, TsFAEC-I5F/TsFAEC-I3R, along with each single primer as a control for non-specific PCR amplification products (Table 2-3). The resulting PCR products were gel-purified before cloning into the pCR®2.1-TOPO® vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

A full-length *faeC* gene was amplified from *Tal. stipitatus* genomic DNA using the TsFAEC-ssN1F forward and TsFAEC-C16R reverse primers, in which *Sna*BI and *Not*I sites have been introduced at the 5' and 3' ends respectively (Table 2-3). DNA amplification was carried out through 30 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 60 °C), and extension (1 min 45 sec at 72 °C), followed by 10 min at 72 °C, using *Vent* DNA polymerase with the 10 X ThermoPol buffer (10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 % Titon X-100). Adenine single-base 3' overhang was added by incubation of the gel-purified PCR product for 10 minutes at 72 °C with 1.5 U *Taq* DNA polymerase, 0.1 volumes 10 X PCR buffer and 500 µM dATP, before cloning into the pCR®4-TOPO® vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

2.4.2.2. RACE-PCR

The RACE technique is based on the RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) and results in the selective ligation of the RNA oligonucleotide (GeneRacer™ RNA Oligo) to the 5' ends of decapped mRNA using the T4 ligase. The GeneRacer™ Kit was purchased from Invitrogen™ Living Science. The following steps were performed according to the manufacturer's procedure.

For cDNA production, 3 µg of total RNA (extracted after 8 hours incubation with SBP) was incubated with 3 U of RNase-free DNase I (Promega), followed by RNA dephosphorylation using the calf intestinal phosphatase (CIP), removal of the mRNA cap structure using the tobacco acid pyrophosphatase (TAP) and ligation of the RNA oligonucleotide using the T4 RNA ligase according to the manufacturer's protocol. The cDNA products of two independent reverse-transcription (RT) protocols were utilised as templates to amplify the *faeC* gene.

First, 1 µl of the GeneRacer™ Oligo dT and 1 µl of deoxynucleoside triphosphate mix (10 mM each) were added to the ligated RNAs (10 µl). The RNAs were incubated at 65 °C for 5 min, chilled on ice for 2 min and centrifuged briefly. The following reagents were added: 4 µl of 5 X first strand buffer (250 mM Tris-HCl, pH8.3, 375 mM KCl, 15 mM MgCl₂), 2 µl DTT (0.1 mM), 1 µl RNaseOUT™ (40 U µl⁻¹), 1 µl SUPERSCRIPT™ II reverse-transcriptase (200 U µl⁻¹). The reaction was incubated at 42 °C for 50 min and inactivated at 70 °C for 15 min. 1 µL of RNase H (2 U µl⁻¹) was added and the reaction was incubated at 37 °C for 20 min. The RT reaction was diluted 4-fold and 1 µl was used as template for PCR.

Second, 1 µl of the GeneRacer™ Oligo dT was added to the ligated RNAs (10 µl). The RNAs were incubated at 75 °C for 5 min and the temperature was dropped to 42 °C. At this temperature the following reagents were added: 1 µl 100 mM dNTPs, 2 µl 10 X RT buffer (250 mM Tris-HCl, pH8.3, 500 mM KCl, 50 mM MgCl₂, 20 mM DTT), 1 µl AMV reverse-transcriptase (5 U µl⁻¹), 4 µl sterile water, 1 µl

RNaseOUT™ (40 U μl^{-1}). The reverse-transcription was performed at 42 °C for 60 minutes and the enzyme was inactivated at 85 °C for 15 min. The RT reaction was diluted 4-fold and 1 μl was used as template for PCR.

Amplification of the 5' cDNA end: PCR was performed in 50 μl reaction using 1 μl SUPERSRIPT™ II RT reaction as template, 1 μl TsFAEC-I13R reverse primer (100 ng μl^{-1}) (Table 2-3), 1 μl GeneRacer™ 5' Nested Primer (81 ng μl^{-1}), 500 mM of each deoxynucleoside triphosphate, 0.1 volume of 10 X PCR buffer 2 and 5 U Taq DNA polymerase. DNA amplification was carried out through 40 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 65 °C), and extension (1 min at 72 °C) followed by 10 min at 72 °C, using Taq DNA polymerase. The PCR reaction was diluted 10-fold and 1 μl used as template in a new PCR reaction with 1 μl TsFAEC-I12R reverse primer (100 ng μl^{-1}) (Table 2-3), 1 μl GeneRacer™ 5' Nested Primer (81.3 ng μl^{-1}). DNA amplification was carried out through 40 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 65 °C), and extension (1 min at 72 °C), followed by a further 10 min extension at 72 °C. The resulting PCR products were gel-purified before cloning into the pCR®4-TOPO® vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

Amplification of the 3' cDNA end: PCR was performed as above using 1 μl AMV-RT reaction as template, 1 μl TsFAEC-I11F forward primer (100 ng μl^{-1}) (Table 2-3) and 1 μl GeneRacer™ 3' Nested Primer (71 ng μl^{-1}). DNA amplification was carried out through 5 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 70 °C), and extension (1 min at 72 °C), followed by 5 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 68 °C), and extension (1 min at 72 °C), followed by 30 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 65 °C), and extension (1 min at 72 °C), followed by 10 min at 72 °C. The PCR reaction was diluted 10-fold and 1 μl used as template in a new PCR reaction with 1 μl TsFAEC-I14F forward primer (100 ng μl^{-1}) (Table 2-3) and 1 μl GeneRacer™ 3' Nested Primer (71 ng μl^{-1}). DNA amplification was carried out through 5 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 70 °C), and extension (1 min at 72 °C), followed by 10

cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 68 °C), and extension (1 min at 72 °C), followed by 25 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 65 °C), and extension (1 min at 72 °C), followed by 10 min at 72 °C. The resulting PCR products were gel-purified before cloning into the pCR®4-TOPO® vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

The complete cDNA sequence was re-amplified by RT-PCR with 1 µl AMV-RT reaction, 1 µl TsFAEC-ssN1F forward primer (1 µg µl⁻¹), 1 µL TsFAEC-C16R reverse primer (1 µg µl⁻¹) (Table 2-3), 500 mM of each deoxynucleoside triphosphate, 0.1 volume of 10 X ThermoPol and 2 U *Vent* DNA polymerase. DNA amplification was carried out through 30 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 61 °C), and extension (1 min 45 sec at 72 °C), followed by 10 min at 72 °C. Since proofreading DNA polymerases such as *Vent* produce blunt DNA ends, gel-purified PCR product were incubated at 72 °C for 10 minutes with 1.5 U *Taq* DNA polymerase, 0.1 volumes 10 X PCR buffer 2 and 500 µM dATP, in order to add a single adenine base to create a 3' overhang, before cloning into the pCR®4-TOPO® vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

2.4.2.3. Site-directed mutagenesis by inverse PCR

During the mRNA reverse transcription reaction errors have been introduced in the *faeC* cDNA nucleotide sequence. In order to eradicate these nucleotide errors we decided to amplify a contiguous coding region for the *Talaromyces stipitatus* ferulic acid esterase gene (*faeC*) from a genomic DNA clone (pCR®4-TOPO®/*faeC*-gene), which has been carefully checked for nucleotide sequence fidelity.

A full-length cDNA of the gene was obtained by inverse-PCR performed on pCR®4-TOPO®/*faeC*-gene plasmid DNA using the TsFAEC-Int-F forward and TsFAEC-Int-R reverse primers positioned on either side of the intron (Table 2-3). The primers were designed to introduce a silent restriction site (*NsiI*) into the self-ligated

inverse-PCR product, which was used as a selective marker to differentiate specific PCR products from the others. DNA amplification was carried out through 10 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 61 °C), and extension (4 min at 68 °C), followed by 15 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 61 °C), and extension (4 min at 68 °C plus 10 sec extra time per cycle), followed by 10 min at 68 °C, using the Expand Long Template PCR System with the 10 X PCR buffer 3 (500 mM Tris-HCl pH 9.2, 140 mM (NH₄)₂SO₄, 22.5 mM MgCl₂, 20 % (v/v) DMSO, 1 % (v/v) Tween[®] 20). The PCR product was then used in Klenow reaction using 45 µl of the PCR reaction, 2 µl Klenow (5 U/µl), 6 µl Klenow buffer 10 X, 6 µl water, 1 µl dNTPs (25 mM) for 10 minutes at room temperature, allowing PCR product extremities to be filled with dNTPs. The Klenow enzyme was inactivated for 10 minutes at 75 °C. DNA was precipitated with 0.1 volumes 3 M sodium acetate pH 5.2 and 2.5 volumes 100 % ethanol, resuspended in 15 µl of sterile distillate water, followed by kinase reaction in order to add a phosphate at the 5' extremities of the PCR product, which will be required for ligation. A kinase reaction was performed using 15 µl of the resuspended klenow reaction, 2 µl kinase buffer 10 X, 1 µl T4 Polynucleotide Kinase (10 U/µl), 2 µl ATP (0.1 mM), incubated for 30 minutes at 37 °C and inactivated for 10 minutes at 65 °C. PCR product DNA was then self-ligated using 5 µl DNA template (from the kinase reaction), 2 µl ligase buffer 10 X, 1 µl ATP (10 mM), 1 µl ligase (4 U/µl), 11 µl water, incubated overnight at 4 °C. The ligated DNA was transformed into *E. coli* TOP10F' cells and plasmid DNA prepared. Restriction digests were performed to select clones that contain the silent restriction site *Nsi*I, which were sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

Table 2-3: Oligonucleotide sequences used to identify the *Tal. stipitatus faeC* gene.

Primer names	Primer nucleotide sequences with their translations [†]
TsFAEC-N1F	5'-GCX AAR GAR ATH CAY ATH CC-3' A K E I H I
TsFAEC-I1F	5'-TAY CAY GGX ACX GCX GAY CC-3' Y H G T A D
TsFAEC-I2F	5'-TGG GTX GAR GAR GGX GTX GCX CC-3' W V E E G V A
TsFAEC-I4F	5'-GAY CCX AAY TGG GAY CCX GC-3' D P N W D P
TsFAEC-I5F	5'-GGX GTX GAR AAY GTX GAY ATG TA-3' G V E N V D M
TsFAEC-I11F	5'-C AGC TGG AGT GGA CAT TTC TAC CC-3' S W S G H F Y
TsFAEC-I14F	5'-A TTT GAG GGT GAT TTA TCC GCT TTC C-3' F E G D L S A F
TsFAEC-ssN1F	5'-AAA <u>ATA CGT ATG</u> ATG TTG ACA AGT GCA ATC C-3' M M L T S A I
TsFAEC-I3R	5'-CCA XGC RTT YTC RTC XGT CCA-3' W A N E D T W
TsFAEC-I13R	5'-AC GAG TTG GGC GGT ATT GAC TTG C-3' L Q A T N V Q
TsFAEC-I12R	5'-AA CTG ACA CCC TGC GAG ACC ACC-3' Q C G A L G G N
TsFAEC-C16R	5'-AA AAG <u>CGG CCG CTA</u> CTA CAA AAT ACA TTT CCA CC-3' * * L I C K W
TsFAEC-Int-F	5'- <u>GC ATC</u> CAG TAT GTA GAC ATG GC-3'
TsFAEC-Int-R	5'- <u>A TCC</u> TGC GAG ACC ACC ATT AC-3'

[†] R: purine; Y: pyrimidine; X: A, C, T, or G; H: A, C, or T and the restriction sites introduced in the primer sequences are underlined (*Sna*BI: TA CGTA; *Not*I: GC GGC CGC; *Xho*I: CTCGAG; *Nsi*I: ATGCAT). * Indicates the stop codon.

2.4.3. Cloning of *Neurospora crassa* *faeD*-3.544 and *faeD*-3.660 coding regions by polymerase chain reaction

As no introns have been detected in the translation of the two *N. crassa* sequences, cDNAs containing native signal sequence were PCR amplified from genomic DNA using specific primers designed on the N and C-termini of the genes (Table 2-4). DNA amplification was carried out through 30 cycles of denaturation (30 sec at 95 °C), annealing (45 sec at 62 °C), and extension (1 min 50 sec at 72 °C), followed by 10 min at 72 °C, using *Pfu* DNA polymerase with the 10 X Cloned *Pfu* buffer (200 mM Tris-HCl pH 8.8, 20 mM MgSO₄, 100 mM (NH₄)₂SO₄, 1 % (w/v) Triton X-100, 1 mg/ml nuclease-free BSA). An adenine single-base 3' overhang was added by incubation of the gel-purified PCR product for 10 minutes at 72 °C with 1.5 U *Taq* DNA polymerase, 0.1 volumes 10 X PCR buffer and 500 µM dATP, before cloning into the pCR®2.1-TOPO® vector and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

Table 2-4: Oligonucleotide sequences used to PCR amplify the cDNA of *N. crassa* *faeD*-3.660 and *faeD*-3.544

Primer names	Primer nucleotide sequences*
Nc3.660-ssNF	5'- AAA <u>ATA CGT ATG</u> TAT CTC TTC GG -3'
Nc3.660-CR	5'- AAA <u>AGC GGC CGC</u> TCA AAA CAT CGC AAG CTG AGG -3'
Nc3.544-ssNF	5'- AAA <u>ATA CGT ATG</u> GCA GGC CTT CA-3'
Nc3.544-CR	5'- AAA <u>AGC GGC CGC</u> TAG TTG AAC CTG CTA AAG AAC -3'

*The restriction sites introduced in the primer sequences are underlined (*Sna*BI: TA CGTA; *Not*I: GC GGC CGC)

2.5. CONSTRUCTION OF FERULOYL ESTERASE WILD TYPE AND MUTANT EXPRESSION VECTORS

2.5.1. Construction of *Escherichia coli* expression vector

N. crassa fae-1 cDNA, which does not contain a signal sequence, was isolated from the cloning vector pCR[®]2.1-TOPO[®]/*fae-1* (section 2.4.1.) by restriction endonuclease digest with *NdeI*-*Bam*HI and ligated into the vector pET3a digested with the same restriction enzymes resulting in pET3a/*fae-1* expression vector .

2.5.2. Construction of *Pichia pastoris* expression vectors

2.5.2.1. Construction to express *Neurospora crassa Fae-1* in *Pichia pastoris*

The *N. crassa fae-1* cDNA fragment containing the native signal sequence was isolated from the pCR[®]2.1-TOPO[®] cloning vector (section 2.4.1.) by restriction digest using *Bam*HI-*Not*I endonucleases and ligated into the *Bam*HI-*Not*I digested pPIC3.5K vector, resulting in pPIC3.5K/*fae-1* expression vector.

2.5.2.2. Construction to express *Neurospora crassa FAED-3.544* and *FAED-3.660* in *Pichia pastoris*

The *N. crassa faeD-3.544* and *faeD-3.660* cDNA fragments were isolated from the cloning vector (section 2.4.3.) by restriction digest using *Sna*BI-*Not*I endonucleases and ligated into the *Sna*BI-*Not*I digested pPIC3.5K vector, resulting in pPIC3.5K/*faeD-3.544* and pPIC3.5K/*faeD-3.660* expression vectors.

2.5.2.3. Construction to express *N. crassa Fae-1* mutants in *P. pastoris*

Site-directed mutagenesis was performed by inverse-PCR on plasmid DNA (pCR[®]2.1-TOPO[®]/*fae-1*, which cDNA contains the native signal sequence, section 2.4.1.) and self-ligation of the inverse-PCR product. Primers (Nc-S118A-F/Nc-S118A-R and Nc-S91A-F/Nc-S91A-R, Table 2-5) were positioned on either site of the mutation sites for serine 118 and serine 91. The oligonucleotides were designed to replace the serine codons with alanine and to introduce a silent restriction site (*KpnI* and *NsiI* for S118A and S91A, respectively) into the self-ligated inverse-PCR product, which was used as a selective marker. DNA amplification was carried out through 10 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 62 °C), and extension (3 min 30 sec at 68 °C), followed by 15 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 62 °C), and extension (3 min 30 sec at 68 °C plus 10 sec extra time per cycle), followed by 10 min at 68 °C, using the Expand Long Template PCR System. PCR reactions were in turn used in Klenow, kinase and ligation reactions as described for the inverse polymerase chain reaction, section 2.4.2.3. Restriction digests were performed to select clones that contain the silent restriction site, which were sequenced before sub-cloning in the *BamHI-NotI* digested expression vector, resulting in pPIC3.5K/*fae-1*-S118A and pPIC3.5K/*fae-1*-S91A vectors.

Table 2-5: Oligonucleotide sequences used to PCR amplify *N. crassa fae-1* mutants

Primer names	Primer nucleotide sequences*
Nc-S118A-F	5'- <u>CC</u> GCC TCG GGC GGC ATG ATG AC -3'
Nc-S118A-R	5'- <u>TAC CCA</u> TGA CAT AGA CGC GCG -3'
Nc-S91A-F	5'- <u>ATT</u> GGGTATCGTTGCCATGGTGAAC-3'
Nc-S91A-R	5'- <u>GCA</u> TCGCCGCGGCTCCGTGC-3'

*The restriction sites introduced in the primer sequences are underlined (*NsiI*: ATGCAT; *KpnI*: GGTACC). Codons altered are in bold.

2.5.2.4. Construction to express *Talaromyces stipitatus* FAEC in *Pichia pastoris*

2.5.2.4.1. pPIC3.5K/*faeC* vector construction

The cDNA fragment, containing the *Tal. stipitatus* FAEC native signal sequence, was isolated from the cloning vector pCR[®]4-TOPO[®]/*faeC* (section 2.4.2.3.) by restriction digest *SnaBI-NotI* and ligated to the pPIC3.5K vector digested with the same restriction enzymes resulting in the pPIC3.5K/*faeC* expression vector.

2.5.2.4.2. pPIC3.5K/ss1-NFS*faeC* and pPIC3.5K/ss1-DDS*faeC* vector constructions

Single stranded DNA was first PCR amplified from pCR[®]4-TOPO[®]/*faeC* plasmid DNA (section 2.4.2.3.) using single primers TsFAEC-ss1-NFS and TsFAEC-ss1-DDS, respectively (Table 2-6), which contain the *N. crassa* Fae-1 signal sequence. Single stranded DNA amplification was carried out through 5 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 59 °C), and extension (3 min 30 sec at 72 °C), followed by the addition of TsFAEC-ss1-F and TsFAEC-C16R primers (Table 2-6), allowing synthesis of double stranded DNA through 25 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 59 °C), and extension (3 min 30 sec at 72 °C), followed by 10 min at 72 °C, using *Pfu* DNA polymerase. The constructions were DNA sequenced before sub-cloning into *SnaBI-NotI* digested pPIC3.5K expression vector, resulting in the pPIC3.5K/ss1-NFS*faeC* and pPIC3.5K/ss1-DDS*faeC* expression constructs.

2.5.2.4.3. pPIC9/ α -NFS*faeC* vector construction

PCR was performed on pCR[®]4-TOPO[®]/*faeC* plasmid DNA (section 2.4.2.3.) using TsFAEC- α -NFS, which contains the *S. cerevisiae* α -factor signal sequence, and TsFAEC-C16R primers (Table 2-6). DNA amplification was carried out through 25 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 61 °C), and extension

(3 min at 72 °C), followed by 10 min at 72 °C, using *Pfu* DNA polymerase. The construction was DNA sequenced before sub-cloning into *SnaBI-NotI* digested pPIC9 vector, resulting in the pPIC9/ α -NFS*faeC*.

2.5.2.5. Construction to express *Talaromyces stipitatus* FAEC mutants in *Pichia pastoris*

Site-directed mutagenesis was performed by inverse-PCR on plasmid DNA (pCR[®]4-TOPO[®]/*faeC*, section 2.4.2.3.) and self-ligation of the inverse-PCR product. Primers (TsFAEC-S166A-F/TsFAEC-S166A-R and TsFAEC-S465A-F/TsFAEC-S465A-R, Table 2-6) were positioned on either side of the mutation sites corresponding to serine 166 or serine 465 respectively (with the first amino acid being the asparagine identified from the N-terminal sequence of the native FAEC). They were designed to replace the serine codons with alanine and to introduce silent restriction sites (*AviII* and *BlnI* for S166A and S465A, respectively) into the self-ligated inverse-PCR product. DNA amplification was carried out through 10 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 59 °C), and extension (4 min at 68 °C), followed by 15 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 59 °C), and extension (4 min at 68 °C plus 10 sec extra time per cycle), followed by 10 min at 68 °C, using the Expand Long Template PCR System. PCR reactions were in turn used in Klenow, kinase and ligation reactions as described for the inverse polymerase chain reaction, section 2.4.2.3. Restriction digests were performed to select clones that contain the silent restriction site, which were then DNA sequenced before sub-cloning in the *SnaBI-NotI* digested expression vector, resulting in the constructs pPIC3.5K/*faeCS166A* and pPIC3.5K/*faeCS465A*.

Table 2-6: Oligonucleotides sequences used to PCR amplify *Tal. stipitatus faeC* mutants and *faeC* appended with various signal sequences

Primer names	Primer nucleotide sequence*
TsFAEC-ss1-NFS	5'-AAA GCT CGA <u>GTA CGT</u> ATG TTG CCC AGA ACA TTG CTC GGG CTC GCC CTC ACC GCG GCC ACA GGC CTC TGT AAC TTC TCA AAT CGA TGC GA-3'
TsFAEC-ss1-DDS	5'-AAA GCT CGA <u>GTA CGT</u> ATG TTG CCC AGA ACA TTG CTC GGG CTC GCC CTC ACC GCG GCC ACA GGC CTC TGT GAT GAT TCG TCC CGC GAG AAC-3'
TsFAEC-ss1-F	5'-AAA GCT CGA <u>GTA CGT</u> ATG TTG C-3'
TsFAEC-C16R	5'-AAA <u>AGC GGC CGC</u> TAC TAC AAA ATA CAT TTC CAC C-3'
TsFAEC- α -NFS	5'-AA <u>GCT CGA GAA</u> AAG AGA GGC TGA AGC TAA CTT CTC AAA TCG ATG CGA CC-3'
TsFAEC-S166A-F	5'- <u>C GCA</u> ACA GGC GGA CGA CAA GGG-3'
TsFAEC-S166A-R	5'- <u>CA</u> GCC GAG ATA GTA TGA TTT G-3'
TsFAEC-S465A-F	5'- <u>CTA GGT</u> AGT GGG CCG GGA GC-3'
TsFAEC-S465A-R	5'- <u>GGC</u> AGC ACC ACG AAT ATA CTC CG-3'

*The restriction sites introduced in the primer sequences are underlined (*Sna*BI: TA CGTA; *Not*I: GC GGC CGC; *Xho*I: CTCGAG; *Nsi*I: ATGCAT; *Avi*II: TGCGCA; *Bln*I: CCTAGG). Codons altered are in bold.

2.6. OVER-EXPRESSION OF RECOMBINANT FERULOYL ESTERASES

2.6.1. Expression in *Escherichia coli* BL21(DE3)pLysS

The target genes were cloned in pET vectors under the control of a strong bacteriophage T7 transcriptional promoter. Transcription may then be induced by providing a source of T7 RNA polymerase from the host cell. For protein production,

a recombinant plasmid carrying the target gene was transferred to the host *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase. Such hosts are lysogens of bacteriophage DE3, a lambda derivative that carries a DNA fragment containing the *lacI* gene and the *lacUV5* promoter directing transcription of the T7 RNA polymerase gene, which is inducible by IPTG. Addition of IPTG to a growing culture of lysogen induces T7 RNA polymerase, which in turn transcribes the target gene under the control of a T7 promoter. Even in the absence of IPTG, there is some expression of T7 RNA polymerase from the *lacUV5* promoter in DE3 lysogens. To control this, host strains such as BL21(DE3)pLysS, which contain a compatible plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase, are employed.

2.6.1.1. *Small-scale expression*

To optimise the expression, cells were grown at 37 °C in 10 ml of LB to an OD₆₀₀ value of 0.3, 0.4 or 0.6 at which point, protein expression was induced by adding 0.4 or 0.1 mM IPTG. The cultures were subsequently incubated at 37 °C, 30 °C or 25 °C for 4 hours. Culture aliquots (1 ml) were removed before and after induction. Cells were collected by centrifugation, resuspended in SDS-loading buffer and proteins were analysed by SDS-PAGE gels (Sambrook *et al.*, 1989), section 2.6.2.6.

2.6.1.2. *Isolation of material from cell fractions*

One millilitre of culture collected after 4 hours induction was centrifuged for 1 minute at 12 000 g and 4 °C. The supernatant was discarded and the pellet resuspended in 100 µl of solution I (20 % (w/v) sucrose, 30 mM Tris-HCl pH 8.0, 1mM EDTA) then incubated on ice for 10 minutes. Cells were pelleted for 1 minute at 12 000g and 4 °C and the supernatant kept as the periplasmic space fraction. Cells were resuspended in 100 µl of solution II (0.1M Tris-HCl pH 8.0), frozen in liquid nitrogen and thawed at 37 °C. This procedure was repeated to lyse the cells. The supernatant obtained after a centrifugation at 12 000 g and 4 °C for 15 minutes was retained as the cytoplasmic fraction. 100 µl of 1 % (w/v) Triton X-100 was added to the pellet and incubated for 10 minutes at 4 °C to solubilise membrane proteins

and inclusion bodies. Protein fractions were analysed by SDS-PAGE gels and the target protein N-terminal amino acid sequence determined using Sequencer Model 473A (Applied Biosystems).

2.6.2. Expression in *Pichia pastoris*

The *Pichia pastoris* strain GS115 possesses a mutation in the gene for histidinol dehydrogenase (*his4*) that prevents synthesis of histidine, which must therefore be added to growth media. The *HIS 4* gene is present in *Pichia* expression vectors and allows the selection of transformants (phenotype His⁺) by their ability to grow on histidine-deficient medium. Induction of expression is driven specifically by methanol, acting at the *AOX1* promoter (P_{AOX1}) present in *Pichia* expression vectors.

2.6.2.1. Preparation of electrocompetent *P. pastoris* cells

Pichia was grown in 5 ml YPD in a 50 ml conical tube at 30 °C overnight. The following day 500 ml of fresh YPD medium in a 2 Litre flask were inoculated with 0.1 - 0.5 ml of the overnight culture and grown overnight at 30 °C with vigorous shaking (250 – 300 rpm) to an OD₆₀₀ between 1.3 and 1.5. Cells were centrifuged at 1 500 g for 5 minutes at 4 °C and the pellet resuspended in 500 ml of ice-cold sterile water. Cells were centrifuged as before and resuspended in 250 ml of ice-cold sterile water. Centrifugation was repeated once again and cells resuspended in 20 ml of ice-cold 1 M sorbitol, followed by a new centrifugation and resuspended in 1 ml ice-cold 1 M sorbitol. Cells were kept on ice and used the same day.

2.6.2.2. Transformation of electrocompetent *P. pastoris* cells

Transformation of *Pichia pastoris* GS115 was performed by electroporation (Becker and Guarente, 1991). For transformation, 1 - 5 µg of vector was linearised with *DraI* restriction enzyme to allow gene replacement at *Pichia pastoris AOX1* gene. 80 µl of *Pichia* cells prepared as described above were mixed with 1-5 µg of linearised DNA (in 5 µl sterile water) and incubated on ice for 5 minutes. Cells were transferred to an ice-cold 0.2 cm electroporation cuvette and pulsed according to the parameters for

yeast suggested by the manufacturer (1.5 kV, 200 Ω , 25 μ F, using a BioRad gene pulser with a pulse controller). Immediately 1 ml of ice-cold 1 M sorbitol was added to the cuvette. Transformed cells were directly plated out by spreading 100 μ l on RDB plates (Regeneration Dextrose Base). Plates were incubated for 2 to 3 days at 30 °C and transformants were selected by their ability to grow at 30 °C on histidine-deficient medium.

2.6.2.3. *Screening transformants*

To assess His⁺ transformants for the Methanol Utilisation phenotype (Mut), colonies were picked up using sterile tooth pick and patched onto a MM (Minimal Methanol) plate and then a MD (Minimal Dextrose) plate in a regular pattern. Normal growth on dextrose and slow growth on methanol is indicative of the Mut^s phenotype.

2.6.2.4. *Small-scale expression*

Single colonies picked from MD plate were used to inoculate 10 ml BMGY (buffered complex glycerol medium) medium. Covered by loose caps, the cultures were incubated at 30 °C with vigorous shaking (>200 rpm) until growth reached saturation (2 days), with an OD₆₀₀ = 10 - 20. Cells were harvested by centrifugation at 4 000 g for 10 minutes at room temperature and resuspended in 2 ml BMMY (buffered complex methanol medium). The cultures were covered with loose caps and incubated at 30 °C with vigorous shaking for 3 days. Methanol (100 %) was added to a final concentration of 0.5 % methanol every 24 hours to maintain induction. The cells were pelleted by centrifugation at 4 000 g and cell culture supernatants analysed by SDS-PAGE for secreted expressed products.

2.6.2.5. *Large-scale expression*

Single colonies on MD plate or frozen stock were used to inoculate 10 ml BMGY media and grown overnight at 30 °C with shaking. From these, 2.5 ml (5 ml) aliquots were used to inoculate 250 ml (500 ml) BMGY media and grown in 1 L (2 L) baffled flasks at 30 °C with vigorous shaking to saturation (OD₆₀₀ = 10-20). Cultures were

harvested by centrifugation at 4 000 g for 10 minutes at room temperature and resuspended in 50 ml (100 ml) BMMY media in 500 ml (1 L) baffled flasks, which were covered with loose caps and incubated with vigorous shaking at 30 °C for 4 days. For each day an aliquot of 1 ml was removed to analyse expression level. Methanol 100 % was added to a final concentration of 0.5 % (v/v) methanol every 24 hours to maintain induction. Cultures were centrifuged at 4 000 g as before and supernatants analysed by SDS-PAGE.

2.6.2.6. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

From Sambrook *et al.*, 1989.

Protein samples were mixed with 0.2 volumes of 5 X Loading Buffer (0.0625 M Tris pH 6.8, 20 % (v/v) glycerol, 0.2 % (w/v) SDS, 20 % (v/v) β -mercaptoethanol, 0.00125 % (w/v) bromophenol blue), heated for 2 minutes and centrifuged for 5 minutes if the samples contained particulate material. Samples were separated on a 12 % (w/v) separating gel with a 5 % (w/v) stacking gel.

A 12 % (w/v) separating gel was prepared by mixing 1.5 M Tris-HCl, pH 8.8, 0.4 % (w/v) SDS solution with 30 % (w/v) acrylamide, TEMED, 10 % (w/v) APS and water so that the final concentrations were 0.375 M Tris-HCl, pH 8.8, 0.1 % SDS, 12 % (w/v) acrylamide, 0.05 % (v/v) TEMED and 0.05 % (w/v) APS. The TEMED and APS were added just before the gel was poured. Approximately 5 ml of separating gel was made for each gel to be poured. Before the separating gel had polymerised it was overlaid with water-saturated butanol. When the gel had set, the butanol was rinsed thoroughly with water and the top of the gel blotted dry using filter paper. A 5 % (w/v) stacking gel was prepared by mixing 0.25 M Tris-HCl, pH 6.8, 0.2 % (w/v) SDS solution with 30 % (w/v) acrylamide, TEMED, 10 % (w/v) APS and water so that the final concentration were 0.125 M Tris-HCl, pH 6.8, 0.1 % (w/v) SDS, 5 % (w/v) acrylamide, 0.1 % (v/v) TEMED and 0.1 % (w/v) APS. Approximately 1 ml of stacking gel was poured on top of the separating gel. The comb was positioned in the stacking gel as soon as possible after pouring. When the stacking gel was polymerised, gels were arranged in pairs in a gel tank electrophoresis. The tank was filled with 1 X running buffer (1 L of 5X running buffer: 7.5 g glycine, 15 g Tris,

5 g SDS in distilled water), the comb was carefully removed and samples were loaded into the wells. Protein samples were separated under 200 V for 45 minutes. Then the separating gel was cut away from the stacking gel, stained in Coomassie Blue Gel Stain for about 1-2 hours (0.25 % (w/v) Coomassie Blue Brilliant Blue R, 10 % (v/v) glacial acetic acid, 25 % (v/v) methanol and water) and destain for about 1 hour (10 % (v/v) glacial acetic acid, 25 % (v/v) methanol and water). Alternatively the gel was electro-blotted onto PVDF membrane for protein sequencing.

2.6.2.7. *Electro-blot for N-terminal sequencing*

After SDS-PAGE, the gel was transferred into electroblotting buffer (25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol) for 30 minutes. Four pieces of Whatman® 3MM paper and one piece of PVDF membrane (polyvinylidene difluoride) cut to the size of the gel were soaked in electroblotting buffer as well as two fibre pads. A "sandwich" was then constructed between the fibre pads starting with two pieces of Whatman® paper on the anode plate, followed by the membrane that had been pre-soaked in methanol, the gel and two further sheets of Whatman® paper. This was placed in the Transblot apparatus, which was filled with electroblotting buffer. Proteins were transferred to the membrane at 4 °C overnight by applying 100 mA. The membrane was removed and stained with in Coomassie Blue Gel Stain for 5 minutes, destained in 50 % (v/v) methanol and rinse with water. The bands to be sequenced were cut from the membrane and sent to Nottingham Queens Medical Centre to be sequenced. N-terminal amino acid sequence was determined using Sequencer Model 473A (Applied Biosystems).

2.6.2.8. *Preparation of frozen stocks*

Single colonies of His⁺ transformants expressing target recombinant protein were cultured overnight in YPD media (10 ml) at 30 °C. The cells were harvested by centrifugation at 500 g and resuspended in YPD containing 15 % (v/v) glycerol at a final OD₆₀₀ of 50 - 100 (approximately 2.5 - 5 x 10⁹ cell ml⁻¹) followed by flash freezing in liquid nitrogen and storage at - 70 °C.

2.7. PURIFICATION AND CHARACTERISATION OF THE RECOMBINANT FERULOYL ESTERASES EXPRESSED IN PICHIA PASTORIS

Large-scale culture was performed in 2 L Erlenmeyer flasks. Cells were grown in 500 ml BMGY pH 6.00 medium at 30 °C for 48 hours (OD₆₀₀ 20-25). The recovered cells were resuspended in 200 ml BMMY pH 6.00 and grown for a further 5 days at 30 °C (200 rpm). Culture was centrifuged at 12 000 g for 15 minutes at room temperature and supernatant recovered. Solid ammonium sulphate was added to a final concentration of 0.6 and 1 M, for FAEC and Fae-1 respectively. The solution was clarified by centrifugation at 10 000g for 15 min at 4 °C. Aliquots of 30 ml were loaded onto a butyl sepharose 4 Fast Flow Hydrophobic Interaction Chromatography column (2.6 x 10 cm) membrane (Amersham Pharmacia Biotech, UK). Unbound material was eluted (5 ml min⁻¹) with 50 mM sodium phosphate buffer, pH 7.0; 1 M ammonium sulphate (buffer A), followed by elution of the bound proteins with a gradient of buffer A - buffer B (50 mM sodium phosphate buffer, pH 7.0). Fractions were collected and assayed for activity against methyl caffeate using a spectrophotometric method (Ralet *et al.*, 1994b). Active fractions were pooled and desalted through a NAP-5 column membrane (Amersham Pharmacia Biotech, UK) into water and a 240 - 400 nm absorbance spectrum recorded.

2.7.1. Estimation of the protein concentration

The extinction coefficient for feruloyl esterase proteins was determined from their amino acid content, and the value (90960 M⁻¹ cm⁻¹ and 51790 M⁻¹ cm⁻¹ for FAEC and Fae-1, respectively) was used to determine the concentration of the samples, following the Beer-Lambert law.

2.7.2. Electrospray ionisation-mass spectrometry

Fae-1 was concentrated 13-fold through a 10 000 MW cut-off ultrafiltration membrane (Amicon) and 3 ml of sample was dialysed against 5 litres of MilliQ

water using a Slide-A-Lyzer cassette (10 000 MW cut-off, Pierce Chemical Co). Electrospray ionisation-mass spectrometry (ESI-MS) of Fae-1 was performed using a Quattro II instrument (Micromass, Manchester, UK).

2.7.3. Feruloyl esterase activity assay

Assays for ferulic acid esterase activity were performed by incubating methyl esterified substrates (MFA, MCA, MSA, MpCA) (Faulds and Williamson, 1991) or feruloylated oligosaccharides Ara₂F ([2-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1,5)-L-arabinofuranose) (Kroon *et al.*, 1997b), in a final volume 0.5 ml for 15 min in 100 mM MOPS buffer pH 6.0 at 37 °C. Agro-industrial waste such as sugar beet pulp, wheat bran or brewer's spent grain (10 mg), were incubated in the presence of feruloyl esterase for 3 hours in 100 mM MOPS buffer pH 6.0 at 37 °C. Reactions were performed in triplicate and terminated by the addition of glacial acetic acid (0.2 ml) and 0.2 ml samples were assayed for phenolic acids by reverse-phase HPLC (Faulds and Williamson, 1994). Controls containing the reaction mixture plus glacial acetic acid were incubated and analysed in identical manner to eliminate interference. One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1 μ mol of free ferulic acid per minute under the defined conditions.

Kinetic constants (k_{cat} , K_m , V_{max}) were calculated from initial rate data using the Michaelis-Menten equation (Equation 2-1).

Equation 2-1:
$$v = (V_{max} \times [S]) / (K_m + [S])$$

Due to substrate inhibition the kinetic constants were determined at low values of substrate (from 0.02 mM to 0.4 mM of substrate) which is permissible when K_i is large compared with K_m . The K_i was estimated from initial rate data determined at various substrate concentrations (from 0.02 mM to 3 mM of substrate), using the equation with respect to a substrate inhibition (Equation 2-2).

Equation 2-2:
$$v = (V_{max} \times [S]) / (K_m + [S] + ([S]^2/K_i))$$

The data were plotted in parallel using Graphit and Excel software. Data curves were fitted using Graphit and/or Excel's solver program modules.

2.7.4. Chlorogenate esterase activity assay

Chlorogenate esterase activity was determined using 5-O-caffeoyl-quinic acid (chlorogenic acid) in a final volume 0.5 ml for 15 min in 100 mM MOPS buffer pH 6.0 at 37 °C. Reactions were terminated by the addition of glacial acetic acid (0.2 ml) and 0.2 ml samples were assayed for phenolic acids by reverse-phase HPLC (Faulds and Williamson, 1994). One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1 μmol of caffeic acid per minute under the defined conditions.

2.7.5. Acetyl esterase activity assay

Acetyl esterase activity was determined using α -naphthyl acetate (0 – 2.5 mM) in 1 ml reaction volume in 100 mM sodium phosphate buffer pH 6.0 at 37 °C, following the increase in absorbance at 235 nm over a 5 minutes period. Acetyl esterase activity was calculated from the initial rate of change of A_{235} using an extinction coefficient for α -naphthyl acetate of 24 000 $\text{M}^{-1} \text{cm}^{-1}$ (Mastropaolo and Yournon, 1981). One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1 μmol of α -naphthol per minute under the defined conditions.

2.7.6. Temperature and pH activity profiles assay

To determine the pH optimum, the recombinant Fae-1 was incubated for 15 minutes at 37 °C with MFA (0.1 mM final concentration in 500 μl volume reaction) in Mc Ilvaine's buffer titrated to a pH within the range 3.0 to 7.5 and in phosphate buffer titrated to a pH within the range 6.0 to 8.0. To determine the temperature optimum, the recombinant Fae-1 was incubated for 15 minutes with MFA (0.1 mM final concentration in 500 μl volume reaction) in 100 mM MOPS pH 6.0, at a temperature range from 20 to 80 °C. The temperature stability of the recombinant Fae-1 was

determined at 60 °C. The enzyme was incubated for up to 60 minutes at 60 °C, aliquots were removed every 10 minutes and assayed for activity against MFA (0.1 mM final concentration in 500 µl volume reaction), for 15 minutes in 100 mM MOPS pH 6.0 at 37 °C. The reactions were stopped by the addition of 0.2 ml glacial acetic acid. Controls containing the reaction mixture plus glacial acetic acid were incubated to eliminate interference. Samples and blanks were centrifuged at 12 000 g for 5 minutes prior to HPLC analysis of the released ferulic acid (Faulds and Williamson, 1994). One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1 µmol of free ferulic acid per minute under the defined conditions.

2.8. DETECTION OF ENZYMATIC ACTIVITIES IN NEUROSPORA CRASSA CULTURE SUPERNATANTS

N. crassa mycelia were cultured in 100 ml Vogel's sucrose medium for 2 days at 30 °C on a flat-bed shaker (200 rpm). Cultures were vacuum filtered through a sterile Buchner funnel, washed with distilled water and aseptically transferred to 500 ml Vogel's supplemented with 2 % sugar beet pulp (w/v) or 2 % wheat bran (w/v). Five milliliter samples of supernatant were removed every 24 hours for a period of 9 days. Protein concentration was determined by the Bradford method (Bradford, 1976) using the Coomassie Protein Assay Reagent supplied by Pierce (USA).

2.8.1. Detection of feruloyl esterase activity

Ferulic acid esterase activities from supernatant samples were assayed spectrophotometrically at 335 nm by the method of Ralet and co-workers (Ralet *et al.*, 1994b). Activity was measured against methyl esters of 4-hydroxy-3-methoxycinnamic (ferulic: MFA), 3,4-dihydroxycinnamic (caffeic: MCA), 3,5-dimethoxy-4-hydroxycinnamic (sinapic: MSA) and 4-hydroxycinnamic (*p*-coumaric: MpCA), 0.1 mM final concentration in 100 mM MOPS buffer pH 6.0, for 5 minutes kinetic at 37 °C, using 20 µl of culture supernatant. One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1

μmol of free ferulic acid per minute under the defined conditions.

2.8.2. Detection of Xylanase activity

Xylanase activity was determined by incubating 20 μl of culture supernatant with 180 μl substrate (1 % (w/v) Birchwood xylan in 10 mM McIlvaine's buffer pH 5.5), for 10 minutes at 30 °C. Culture supernatant boiled samples were used as controls. Reactions were stopped with addition of DNS reagent (dinitrosalicylic acid, 0.3 ml), and the amount of reducing sugars released measured spectrophotometrically at 550 nm from a standard curve as xylose equivalents (Bailey *et al.*, 1992; Miller, 1959). One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1 μmol of xylose per minute under the defined conditions.

2.8.3. Detection of pectinase activity

Pectinase (polygalacturonase) activity was determined based on the method described by Bailey and collaborators (Bailey *et al.*, 1992). Culture supernatant (20 μl) was incubated with 1 % (w/v) polygalacturonic acid in 0.1 M sodium acetate buffer pH 5.0, for 10 minutes at 37 °C. Culture supernatant boiled samples were used as controls. Reactions were stopped with addition of DNS reagent (dinitrosalicylic acid, 0.3 ml), and the amount of reducing sugars released measured spectrophotometrically at 550 nm from a standard curve as galacturonic acid equivalents (Miller, 1959). One unit of activity (1 U) is defined as the amount of enzyme (mg) or millilitre of culture supernatant releasing 1 μmol of galacturonic acid per minute under the defined conditions.

2.9. PHYLOGENETIC ANALYSES

Multiples alignment of sequences or domains showing feruloyl esterase activity as well as sequence related enzymes such as lipases and construction of neighbor-joining phylogenetic tree (Saitou and Nei, 1987), were performed with the CLUSTAL W program available at www.ebi.ac.uk/clustalw/ (Thompson *et al.*,

1994). The matrices used for the multiple sequence alignment was the BLOSUM matrices recommended for amino acid sequences alignments (Henikoff and Henikoff, 1992). The gap penalties were set for the default values; the gap separation penalty value was 8, the penalty value for extending a gap was 0.05 and the penalty value for opening a gap was 10.

CHAPTER 3. CLONING OF A FERULOYL ESTERASE FROM *TALAROMYCES STIPITATUS*

3.1. INTRODUCTION

In order to expand the range of available feruloyl esterases, knowing that those characterised so far have demonstrated quite diverse properties, we wished to pursue a limited number of species that constitute a rich source of these enzymes. Following the discovery of feruloyl and *p*-coumaroyl esterase activities in the culture supernatant of the thermophilic aerobic fungus *Talaromyces emersonii* (Tuohy *et al.*, 1994a), we decided to look at the production of feruloyl esterases by representatives of the *Talaromyces* genus. The aim was to purify and characterise Type-A (active on methyl sinapate and FAXX) and Type-B esterases (active on MCA, MpCA and Ara₂F) from a mesophilic (*Tal. stipitatus*) and thermophilic (*Tal. thermophilus*) strain of *Talaromyces*. Initial studies carried out at IFR prior to the start of this project suggested that these species produced feruloyl esterase activities in their culture supernatants in response to plant cell wall materials. However, later attempts to identify and isolate these activities from *Tal. thermophilus* when grown on sugar beet pulp were unsuccessful.

3.2. FERULOYL ESTERASE ACTIVITIES IN CULTURE SUPERNATANT OF *TALAROMYCES STIPITATUS*

These initial experiments were carried out by Kroon P.A., Garcia-Conesa M.-T., and Faulds C.B. at IFR, Norwich, and are summarised here to provide a basis for the cloning experiments presented. Currently this information does not appear in any form elsewhere.

3.2.1. Detection of feruloyl esterase activities

Culture supernatants of *Talaromyces stipitatus* strain CBS 375.48 were analysed for feruloyl esterase activities with synthetic substrates after seven days of growth on plant cell wall materials. *Tal. stipitatus* was grown in liquid culture in the presence of sugar beet pulp (2 %) to produce extracellular feruloyl esterase activities against the synthetic substrates MCA (27 U L^{-1}) and MSA (15 U L^{-1}). Growth on wheat bran (2 %) produced extracellular feruloyl esterase activities against MCA (7 U L^{-1}) and MSA (17 U L^{-1}).

3.2.2. Isolation of three discrete feruloyl esterases

Following the detection of feruloyl esterase activities in culture supernatants of *Tal. stipitatus* grown with sugar beet pulp (SBP) or wheat bran (WB), purification of the putative feruloyl esterases was undertaken. These activities could be resolved into three individual esterases. Growth of *Tal. stipitatus* on wheat bran resulted in the purification of one feruloyl esterase presenting the characteristics of a Type-A feruloyl esterase (activity against MSA and release of DiFA, Table 3-1) (Kroon *et al.*, 1997a; Kroon *et al.*, 1999; Williamson *et al.*, 1998a), whereas *Tal. stipitatus* grown on sugar beet pulp allowed the purification of two feruloyl esterases. One of these exhibited the characteristics of a Type-B ferulic acid esterase (activity against MCA but not on MSA, Table 3-1) (Kroon *et al.*, 1996; Williamson *et al.*, 1998a), while the other exhibited activity against MCA and MSA (Table 3-1). This enzyme displayed composite Type-A and Type-B properties and has since been designated as Type-C. Further investigation of this composite activity suggested that the Type-C enzyme is an example of a new type of fungal feruloyl esterase with an hitherto unseen ability to hydrolyse a broad spectrum of methyl hydroxycinnamates (Table 3-1). TsFAEC (Type-C from *Tal. stipitatus*) was found to display similar rates of activity against four substrate hydroxycinnamic acid esters, with rates 5-fold higher than obtained for TsFAEB (Type-B from *Tal. stipitatus*) against MCA. The only other reported esterase that exhibits such a broad spectrum of activity against methyl hydroxycinnamates is a modular acetylsterase (XYLD) from the bacterium *Pseudomonas fluorescens* subsp. *cellulosa* (Faulds *et al.*, 1995b; Ferreira *et al.*,

1993). However, XYLD is reported to exhibit significantly lower activities against synthetic methyl esters compared to the activities observed for TsFAEC. However, TsFAEC unlike XYLD did not release diferulates from either wheat bran (WB) or brewer's spent grain (BSG) in cooperation with xylanases (Bartolome *et al.*, 1997a).

Tal. stipitatus Type-A (TsFAEA) and Type-B (TsFAEB) feruloyl esterases have approximate molecular weights of 35 kDa, and pIs of 5.3 and 3.5, respectively. *Talaromyces stipitatus* Type-C (TsFAEC), however, has a molecular weight of 66 kDa and pI of 4.6 (Table 3-1).

Table 3-1: Substrate specificity of the purified TsFAEC, TsFAEB, and TsFAEA feruloyl esterases from *Tal. stipitatus*, in comparison with other known fungal feruloyl esterases, AnFAEA, AnFAEB from *A. niger* (Faulds and Williamson, 1994^a; Kroon *et al.*, 1996^b; Kroon *et al.*, 1999^c; Ralet *et al.*, 1994b^d) and PfFAEB from *Penicillium funiculosum* (Kroon *et al.*, 2000^e).

	TsFAEC	TsFAEB	TsFAEA	AnFAEA ^{a, c, d}	AnFAEB ^{b, c, d}	PfFAEB ^e
MW	66000	35000	35000	36000	75800	53000
pI	4.6	5.3	3.5	3.3	4.8	6.0
MCA [†]	49.3	10.2	0.43	n.d.	96.9	31.7
MSA [†]	69.7	n.d	134.7	156	n.d.	n.d.
MpCA [†]	64.5	13.5	1.72	0.07	84.2	36.1
MFA [†]	68.5	12.2.	84.3	67.2	22.4	22.9
Ara ₂ F [†]	57.5	6.8	n.d.	n.d.	8.5	98.1
FAXX [†]	69.6	11.5	302.4	114	5.4	24.6
Release of						
DiFA [‡]	n.d.	n.d.	+	+	n.d.	n.d.

n.d.: not detected; [†]: specific activity expressed as U mg⁻¹ of protein, [‡]: qualitative determination

3.3. AMINO ACID SEQUENCES OF THE PURIFIED FERULOYL ESTERASES

N-terminal amino sequences were obtained for all three proteins and internal peptide sequences were obtained for the Type-B and putative Type-C enzymes. Sequence identity searches were performed using the BLAST-P program in order to identify related sequences available from the database at the time of the obtaining of the amino sequence data.

The N-terminal sequence of the Type-A *Tal. stipitatus* feruloyl esterase shows high sequence identity (96 %) with AnFAEA from *A. niger* (de Vries *et al.*, 1997), which is consistent with the Type-A designation based on substrate utilisation (Table 3-2).

The N-terminal sequence of TsFAEB showed 94 % identity to PfFAEB from *P. funiculosum* (Kroon *et al.*, 2000) (Table 3-2). Two internal sequences also show significant identity with sequences present in *P. funiculosum* FAEB (85 % and 88 % identity, respectively). These data suggest that TsFAEB is indeed a Type-B feruloyl esterase, and are consistent with the designation made on the basis of substrate recognition.

N-terminal and internal peptide sequences were obtained from TsFAEC. Results of a similarity search showed no significant alignments for the N-terminal or internal sequences obtained from TsFAEC. A total of 106 residues were determined, representing around 20 % of the total sequence.

Table 3-2: N-terminal peptide sequence identity between *Tal. stipitatus* feruloyl esterases and other fungal feruloyl esterases.

Enzyme	N-terminus peptide sequence	Accession Number
TsFAEA	ASTQGI SEDLYNRLVEMATI IQAAYADL-(N/L)	-
AnFAEA	ASTQGI SEDLYNRLVEMATISQAAYADLCN	AF361950
TsFAEB	ASLTQVSNFGDNPGSLQMYTYVPN NLASKPAIIVAMHP	-
PfFAEB	ASLTQVNNFGDNPGSLQMYTYVPN KLASKPAIIVAMHP	AJ291496
TsFAEC	NFSNR-DQLAKEIHIPNVT	AJ505939

[-]: no peak detected; [()]: uncertainty

3.4. CLONING OF FAEC GENE CODING FOR A TYPE-C FERULOYL ESTERASE FROM TALAROMYCES STIPITATUS

Three feruloyl esterases have been purified from *Tal. stipitatus*. In contrast to TsFAEA and TsFAEB, which demonstrated sequence identity with other fungal esterases, TsFAEC appeared to differ from all other known feruloyl esterases in terms of its peptide sequence as well as its substrate utilisation profile. We therefore decided to try and clone the corresponding gene for TsFAEC in order to provide a full description of the new enzyme. All the following experiments were performed at Nottingham during the course of my studies.

Degenerate oligonucleotides were designed based on the peptide sequences obtained by protein sequencing of the purified TsFAEC. Several PCR reactions were performed on *Tal. stipitatus* genomic DNA, using different primer pairs, under various annealing conditions (Table 3-3) (Chapter 2, section 2.4.2.1.). Six amplified DNA fragments of variable sizes were gel-purified, cloned into the plasmid vector

pCR[®]2.1-TOPO[®] and sequenced. A specific DNA fragment of 1500 bp was amplified using the N-terminal forward primer (TsFAEC-N1F) and the internal reverse primer (TsFAEC-I3R) (Table 3-3). All the internal peptide sequences determined by protein sequencing featured in the translation of this 1500 bp fragment. The predicted protein sequence from this fragment was compared with other protein sequences available in the databank using the BLAST-X and BLAST-P programs. The only significant match found at that time was: 24 % identity and 38 % of similarity with an *Aspergillus oryzae* tannase (Hatamoto *et al.*, 1996). The *faeC* gene fragment was used subsequently to probe colony and plaque lifts of genomic and cDNA libraries, and to probe Southern and Northern blots.

A commercial decision was made to have a custom cDNA library constructed for *Tal. stipitatus* cultured on sugar beet pulp. RNA was isolated using Sokolovsky method after 6 hours growth on pre-digested sugar beet pulp and sent for cDNA cloning. The resulting plasmid cDNA library was screened for a full-length cDNA of the TsFAEC reading frame. However, despite screening 6,000 colonies no such cDNA clone could be recovered. Moreover, PCR amplification with the TsFAEC-N1F and TsFAEC-I3R primer pair using the library DNA as template, did not produce an amplification product. It was concluded that the cDNA sequence of FAEC was not present in this library.

We therefore produced a partial *Sau3A* genomic DNA library in phage lambda to attempt to clone the remaining *faeC* gene sequences. Approximately 50,000 plaques were screened using the *faeC* gene probe. This screen identified a single hybridising plaque, which was plaque-purified and plasmid excised. However, the resulting clone unfortunately contained no further sequence information.

Another approach was to produce a size-restricted genomic library. The library was constructed based on the results of *faeC* hybridising restriction fragments identified from a Southern blot with *Tal. stipitatus* genomic DNA. *EcoRI* restriction of genomic DNA produced *faeC* hybridising fragments of 6 and 3 kb. Genomic DNA restriction digested with *EcoRI* was separated on a preparative agarose gel and the

corresponding sized DNA fragments excised. This DNA was recovered and cloned into lambda ZAP Express[®] and the resulting mini-library screened with the *faeC* gene probe. The required *EcoRI* fragment was not cloned using this approach.

As an alternative to direct cloning a PCR approach was adopted.

Table 3-3: Degenerate oligonucleotide primers designed based on the peptide sequence obtained for TsFAEC from *Tal. stipitatus*.

Peptide sequence	Primer name	Degenerate oligonucleotide primer sequence*
NFSNR-DQLAKEIHIPNVT	TsFAEC-N1F	5'-GCX AAR GAR ATH CAY ATH CC-3'
VLTYHGTADPIITGE(T/E)SK	TsFAEC-I1F	5'-TAY CAY GGX ACX GCX GAY CC-3'
WVEEGVAPEYIR	TsFAEC-I2F	5'-TGG GTX GAR GAR GGX GTX GCX CC-3'
NVYVGPG?WTDENAWK(K)	TsFAEC-I3F	5'-TGG ACX GAY GAR AAY GCX TGG-3'
NVYVGPG?WTDENAWK(K)	TsFAEC-I3R	5'-CCA XGC RTT YTC RTC XGT CCA-3'
YVVFSDPNWDPATLNAQDYE	TsFAEC-I4F	5'-GAY CCX AAY TGG GAY CCX GC-3'
MQPGVENVDMYITYNGDPFA	TsFAEC-I5F	5'-GGX GTX GAR AAY GTX GAY ATG TA-3'
MQPGVENVDMYITYNGDPFA	TsFAEC-I5R	5'-C RTT YTC XAC XCC XGG YTG CAT-3'

*R: purine; Y: pyrimidine; X: A, C, T, or G; H: A, C, or T. The nucleotide sequences correspond with the reverse translations of the amino acid sequences entered in bold (Chapter 2, section 2.4.2.1.).

3.4.1. RACE-PCR

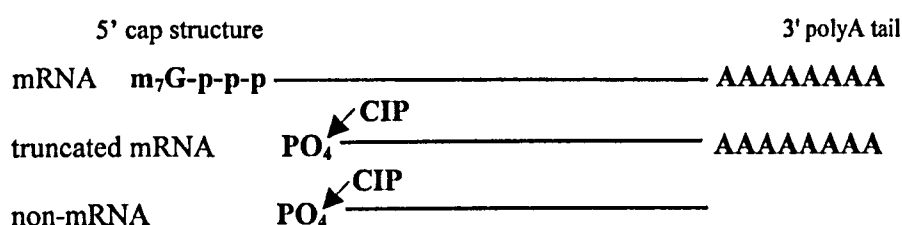
RACE-PCR stands for Rapid Amplification of cDNA Ends - Polymerase Chain Reaction. The RACE technique is based on the RNA ligase-mediated (RLM-RACE) rapid amplification of cDNA ends and is the result of selective ligation of the

RNA oligoribonucleotide (GeneRacer™ RNA Oligo) to the 5' ends of decapped mRNA using the T4 ligase (Chapter 2, section 2.4.2.2.).

3.4.1.1. Strategy employed for RACE

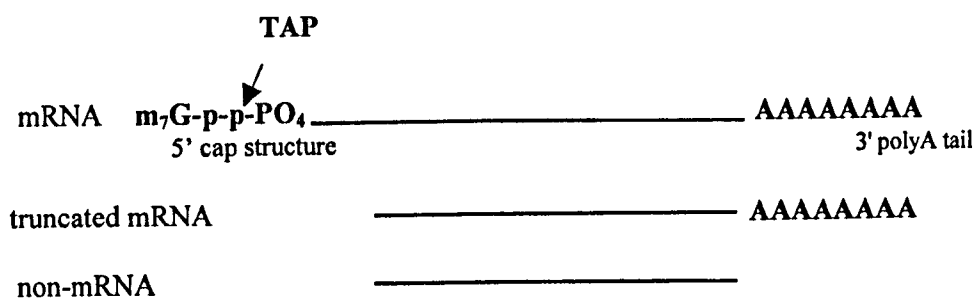
3.4.1.1.1. Dephosphorylation of RNA

Total RNAs were treated with calf intestinal phosphatase (CIP) to remove the 5' phosphates. This procedure prevents non-messenger RNAs and truncated mRNAs from subsequent ligation with the GeneRacer™ RNA Oligo. CIP is ineffective on capped mRNAs.



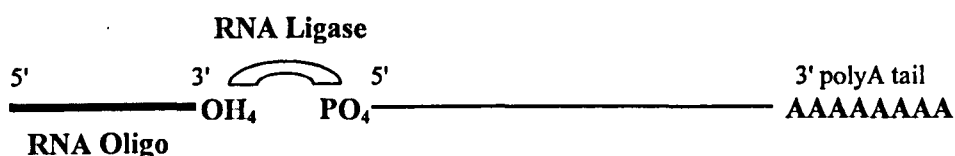
3.4.1.1.2. Removal of the mRNA cap structure

The dephosphorylated RNAs were treated with the tobacco acid pyrophosphatase (TAP) to remove the 5' cap structure from intact, full-length mRNA. This treatment leaves a 5' phosphate required for ligation to the GeneRacer™ RNA Oligo.

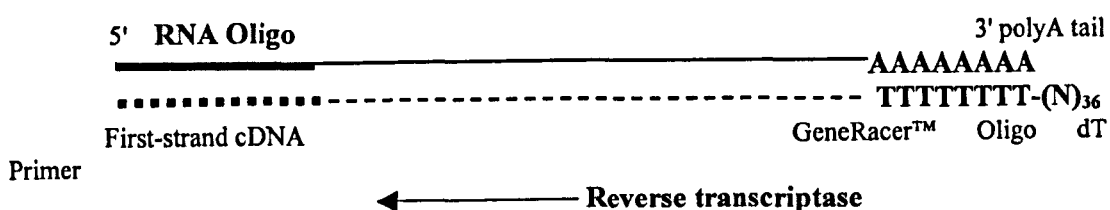


3.4.1.1.3. Ligation of the RNA oligo to decapped mRNA

The Gene Racer™ RNA Oligo was ligated to the 5' end of the mRNA using the T4 RNA ligase. The Gene Racer™ RNA Oligo provides a known priming site for GeneRacer™ PCR primers after the mRNA is transcribed into cDNA.

3.4.1.1.4. Reverse transcription of the mRNA

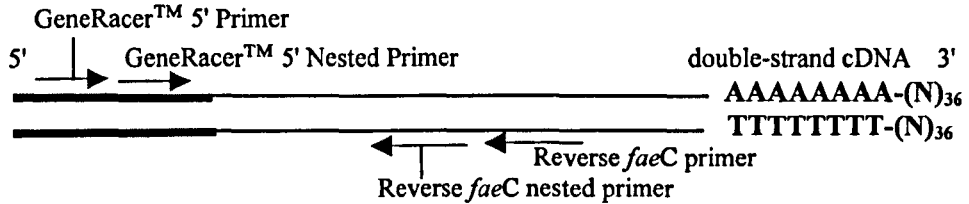
The ligated mRNAs were reverse-transcribed using AMV-RT or SUPERSCRIPT™ II RT, and the GeneRacer™ Oligo dT Primer to create RACE-ready cDNA with known priming sites at the 5' and 3' ends.

3.4.1.1.5. Amplification of cDNA ends

The 5' end was amplified using a reverse *faeC* specific primer and the geneRacer™ 5' Primer (homologous to the GeneRacer™ RNA Oligo). Only mRNA that has the GeneRacer™ RNA Oligo ligated to the 5' end and is completely reverse-transcribed

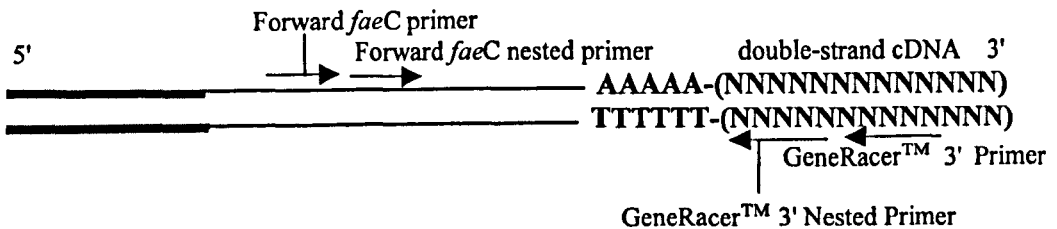
will be amplified by PCR. Additional amplifications were performed with nested primers.

3.4.1.1.5.1. 5'-end amplification



The 3' end was amplified using a forward *faeC* specific primer and the geneRacer™ 3' Primer (homologous to the GeneRacer™ Oligo dT Primer). Only mRNA that has a polyA tail and is reverse-transcribed will be amplified using this strategy. Additional PCR amplifications were performed with nested primers to gain yet further specificity.

3.4.1.1.5.2. 3'-end amplification



3.4.1.2. Extraction of *Tal. stipitatus* RNA

RNAs were extracted from *Tal. stipitatus* following 8 hours of incubation with sugar beet pulp (SBP) pre-digested media. At this stage of incubation we hoped the production of specific *faeC* messengers would be significant. The RNAs were treated with DNase to eliminate genomic DNA contamination and the integrity of the RNAs

after DNase treatment were checked using agarose gel electrophoresis, before commencing the RACE reaction (Figure 3-1).

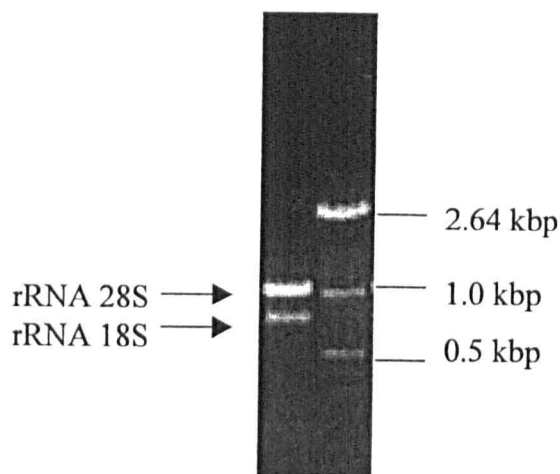


Figure 3-1: *Tal. stipitatus* ribosomal RNA visualised on an agarose gel following DNase treatment.

3.4.1.3. Reverse-transcription of *Tal. stipitatus* mRNA

The reverse transcription was performed either using the SUPERSCRIPT™ II Reverse-Transcriptase (used for the RACE-5' reaction) or the AMV Reverse-Transcriptase (used for the RACE-3' reaction) as described in Chapter 2, section 2.4.2.2.

Before using the newly synthesised cDNA in the RACE-PCR reaction, a PCR control was performed using internal primers specific to *faeC* (TsFAEC-I11F and TsFAEC-I13R, Table 2-3) on both *Tal. stipitatus* cDNA and mRNA templates (Figure 3-2). The purpose of this control was to eliminate the possibility that specific amplification of the *faeC* fragment could arise due to template contamination with genomic DNA. If there was genomic contamination then the expectation would be that a specific *faeC* fragment would be amplified using both the mRNA and cDNA

templates. The experiment showed that genomic DNA did not contaminate the mRNAs as no amplification product was visible before reverse transcription of the mRNA template. A 240 bp *faeC* fragment was only visible when amplified from the *Tal. stipitatus* oligo dT primed cDNA (and from *Tal. stipitatus* genomic DNA, included as a positive control of the amplification process). The PCR-amplification of this specific product also confirms that *faeC* mRNA was present in the mRNA population at 8 hours post-incubation of *Tal. stipitatus* with sugar beet pulp.

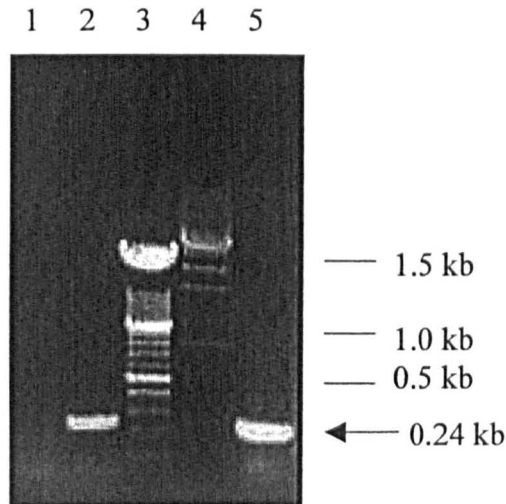


Figure 3-2: PCR controls were performed using internal primers specific to *faeC*, on *Tal. stipitatus* mRNA, cDNA, and genomic DNA templates. Lane 1: PCR performed on mRNA template; lane 2: PCR performed on cDNA template; lane 3: 1 kb DNA ladder; lane 4: 100 bp DNA ladder; lane 5: PCR performed on *Tal. stipitatus* genomic DNA (240 bp).

3.4.1.4. RACE-PCR to amplify *faeC* cDNA ends

The 1500 bp nucleotide sequence data permitted the design of specific nested primers, which were used to amplify *faeC* cDNA ends using the RACE-PCR technique.

3.4.1.4.1. Amplification of *faeC* cDNA 5'-end

The GeneRacer™ 5' Nested Primer and a reverse specific *faeC* primer (TsFAEC-I13R, Table 2-3) were used on the cDNA template to amplify the 5'-end of *faeC* cDNA. PCR with *Tal. stipitatus* genomic DNA using internal *faeC* primers was performed as a positive control of the amplification process. Doublet bands of approximate size 1.1 kb resulted from the cDNA 5'-ends PCR amplification (Figure 3-3). This PCR reaction was utilised as a template for a second PCR amplification using nested primers (GeneRacer™ 5' Nested Primer and TsFAEC-I12R, Table 2-3). The products of the nested PCR could be visualized as a doublet of 480 bp and 460 bp following agarose gel electrophoresis (Figure 3-4). These bands were gel-purified and cloned in pCR® 4-TOPO® vector for DNA sequencing. The sequencing reaction confirmed that both fragments correspond to the N-terminal sequence of the *Tal. stipitatus* FAE C gene. However, a difference of 20 bp at the 5'-ends of the sequences was observed between the two fragments. This could be explained if a proportion of the truncated mRNA population has not been dephosphorylated during the first step of the RACE reaction. Analyses of the nucleotide sequences also revealed that the 5'-cDNA sequence contained 75 bp of non-translated leader sequence and a further 72 bp encoding a peptide signal sequence.

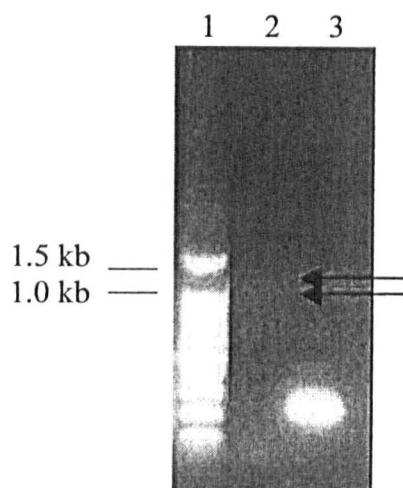


Figure 3-3: Amplification of *faeC* cDNA 5'-end. PCR reaction was performed on *Tal. stipitatus* cDNA template, using the GeneRacer™ 5' Nested Primer and a reverse *faeC* primer (TsFAEC-I13R). Lane 1: 100 bp DNA ladder; lane 2: PCR performed on *Tal. stipitatus* cDNA resulting in doublet bands of 480 bp and 460 bp; lane3: PCR control performed on *Tal. stipitatus* genomic DNA (240 bp).

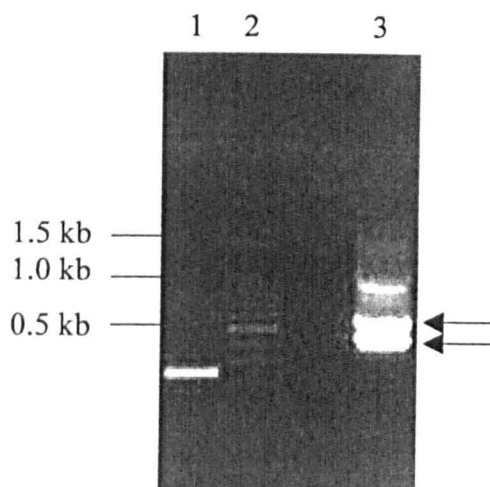


Figure 3-4: Amplification of the *faeC* cDNA 5'-end by nested PCR. PCR was performed using the GeneRacer™ 5' Nested Primer and a reverse *faeC* nested primer (TsFAEC-I12R). Lane 1: PCR control performed on *Tal. stipitatus* genomic DNA (240 bp); lane 2: 100 bp DNA ladder; lane 3: 5'-end *faeC* nested PCR resulting in doublet bands of 480 bp and 460 bp.

3.4.1.4.2. Amplification of *faeC* cDNA 3'-end

PCR amplification was performed on *Tal. stipitatus* cDNA using a forward *faeC* specific primer (TsFAEC-I11F, Table 2-3) and the GeneRacer™ 3' Primer. PCR with *Tal. stipitatus* genomic DNA using internal *faeC* primers was performed as a positive control of the amplification process as noted previously. From this first PCR reaction no distinctive band was observed upon agarose gel electrophoresis, rather just a smear (Figure 3-5). Nevertheless, this PCR reaction was then in turn used as template for a nested PCR (GeneRacer™ 3' Nested Primer and TsFAEC-I14F, Table 2-3), which produced a major band at 740 bp (Figure 3-6). The 740 bp fragment was gel-purified and cloned into pCR® 4-TOPO® for DNA sequencing. The sequencing reaction confirmed the fragment corresponds to the C-terminal sequence of the *Tal. stipitatus faeC* gene. The sequence was in frame with the *faeC* sequence we had determined previously, and contains a termination codon plus 200 bp of downstream non-coding sequence followed by a 25 bp poly-A tail.

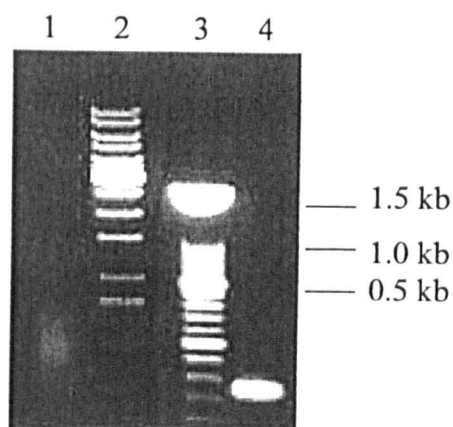


Figure 3-5: Amplification of the *faeC* cDNA 3'-end. PCR was performed on *Tal. stipitatus* cDNA, using the GeneRacer™ 3' Primer and a forward *faeC* primer (TsFAEC-I11F). Lane 1: PCR performed on *Tal. stipitatus* cDNA resulting in a DNA smear; lane 2: 1.0 kb DNA ladder, lane 3: 100 bp DNA ladder; lane 4: PCR control performed on *T. stipitatus* genomic DNA (240 bp).

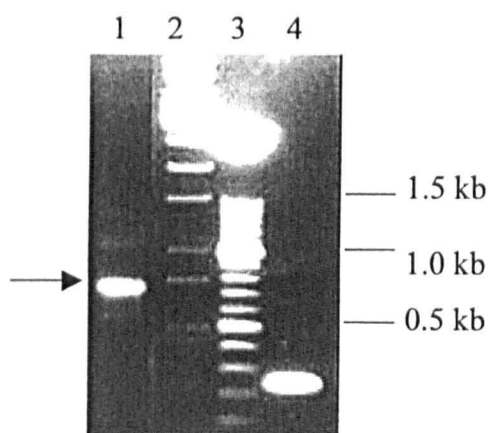


Figure 3-6: Amplification of the *faeC* cDNA 3'-end by nested PCR. PCR was performed using the GeneRacer™ 3' Nested Primer and a forward *faeC* nested primer (TsFAEC-I14F). Lane 1: nested PCR resulting in a product of 740 bp; lane 2: 1.0 kb DNA ladder, lane 3: 100 bp DNA ladder, lane 4: PCR control performed on *T. stipitatus* genomic DNA (240 bp).

3.4.2. Sequence analysis

AMV-RT cDNA products and *Tal. stipitatus* genomic DNA templates were used to PCR amplify the *faeC* coding sequences using the primer pair TsFAEC-ssN1F/TsFAEC-C16R (Table 2-3). The amplified cDNA sequence contained a 1590 bp open reading frame encoding a 531 amino acid residue protein in which 19-25 amino acids encoded a signal peptide (Figure 3-7, GenBank Accession Number: AJ505939). From the mature N-terminal peptide sequence determined from native TsFAEC, a pre-sequence of 25 amino acids may be predicted. However, the signal peptidase cleavage site predicted by the computer program SignalP, is only 19 amino acids long, which would leave the possibility that a further six amino acids are trimmed post-secretion (Nielsen *et al.*, 1997). The predicted molecular mass for the mature protein is 55 340 Da. Comparison of the cDNA and genomic nucleotide sequences revealed the presence of a single intron of 75 bp, which is in frame with the translated open reading frame (Figure 3-7).

The translated sequence of the gene encoding TsFAEC was compared with protein sequences available in the public databank (nr) using BLAST-X. At the time of cloning, only the *A. oryzae* tannase sequence exhibited sequence identity (24 %) with TsFAEC (Ihatamoto *et al.*, 1996). Later database searches revealed 47 % identity with a feruloyl esterase sequence (AnFAEB) recently reported from *A. niger* (de Vries *et al.*, 2002b), 28 % with a tannase (EC 3.1.1.20) reported from *Xanthomonas campestris* (da Silva *et al.*, 2002) and 23 % with a chlorogenate esterase reported from *Acinetobacter* sp. ADP1 (Accession Number: AAL54855). However, the highest identity (62 %) noted at the time of writing is with an unnamed protein product from *Aspergillus oryzae* (Accession Number: CAD28402) lodged in the patent database.

Analysis of the primary sequences revealed the presence of two motifs (GX SXG) characteristic of the serine esterase family (Figure 3-7) (Blow, 1990; Brenner, 1988; Kroon *et al.*, 2000; Warshel *et al.*, 1989). The first motif (GCSTG) is centred on serine 166. Based on the conservation of the sequence environment around serine

166 with the AnFAEB and *Aspergillus oryzae* tannase sequences, this residue could well represent the catalytic nucleophile. A second motif can also be observed close to the C-terminus of the protein (GASLG), which is centred on serine 465 but this motif is not conserved within the tannase or the AnFAEB sequences. The translated sequence does not feature a recognisable cellulose-binding domain as has been observed with the Type-B feruloyl esterase from *P. funiculosum* (Kroon *et al.*, 2000).

Figure 3-7: Nucleotide and deduced amino acid sequences of the *faeC* gene from *Talaromyces stipitatus*. The peptide signal deduced from the N-terminal peptide sequence of TsFAEC is shown in italic and the hash symbol positions the cleavage site predicted by the computer program SignalP. The peptide sequences obtained by protein sequencing of native *faeC* are underlined. The 75 bp intron is shown in lower case. The two sites featuring the consensus motif (GX SXG) containing the active-site serine are shown in grey boxes with the putative active-serine in bold. Potential N-linked glycosylation sites (occurring at the Asn residue within the Asn-X-Thr/Ser/Cys- sequence, where X can be any residue except proline) are shown in reverse field. An asterisk represents the stop codon.

GCTTTTTCTTGGCTTTTTCTTCCACTATGCTTCATCGCGTGGGAGAAGC	50
AAAACGAATCCGTGAACATCCGAGATGATGTTGACAAGTGCAATCCTCCT	100
<i>M M L T S A I L L</i>	-9
CCTGACTCTCGGGGTCCAGCTGAGTCACGCAGATGATTCGTCCCGCGAGA	150
<i>L T L G V Q L S H A # D D S S R E N</i>	1
ACTTCTCAAATCGATGCGACCAGCTGGCTAAGGAGATCCATATTCCCAAC	200
<u>F S N R C D Q L A K E I H I P</u> *	17
GTAAGTGTAACTTTGTGGAATATGTTGCCAATGGCACCAACGTCACACT	250
V T V N F V E Y V A N G T N V T L	34
TGCTGACAATCCGCCTTCATGCGGGCAGTCCAATCAAGTGGTCTCTGGCGG	300
A D N P P S C G Q S N Q V V L A D	51
ATTTGTGTCGAGTGGCTATGGAGGTTACAACCTCCAACCAGAGTCAGATC	350
L C R V A M E V T T S N Q S Q I	67

ACCTTGGAGGCATGGTTTCCGAAAACTACACTGGAAGGTTTCTGAGTAC 400
 T L E A W F P E **N Y T** G R F L S T 84
 CGGTAATGGTGGTCTCGCAGGGTgtcagttccttcctccctccctatcttg 450
 G N G G L A G C 92
 tgggtgtgcattgcggtgaaacaaattaacaccatcacccggagattagGT 500
 ATCCAGTATGTAGACATGGCATATGCGTCGTCAATGGGATTTGCGACAGT 550
 I Q Y V D M A Y A S S M G F A T V 109
 AGGCGCAAATGGGGGCCACAATGGCACCTCCGGAGAATCCTTCTACCACA 600
 G A N G G H **N G T** S G E S F Y H N 126
 ACCCGGACATCGTAGAGGATCTCTCTTGGCGATCAGTGCATACCGGTGTC 650
 P D I V E D L S W R S V H T G V 142
 GTCGTGGGCAAAGAATTGACCAAAAAATCTACCACGAAGGATTCCACAA 700
 V V G K E L T K K F Y H E G F H K 159
 ATCATACTATCTCGGCTGCTCTACAGGCGGACGACAAGGGTTCAAGGCAG 750
 S Y Y L **G C S T G** G R Q G F K A V 176
 TCCAAGAATTTGTGCATGACTTTGACGGCGTGGTCGCAGGGTGTCCGGCG 800
 Q E F V H D F D G V V A G C P A 192
 TTCAACTTTGTAAATCTCAACAGCTGGAGTGGACATTTCTACCCTATCAC 850
 F N F V N L N S W S G H F Y P I T 209
 AGGCAACTCATCCGCAGACACGTTCTGACAACCGCACAGTGGACACTAG 900
 G N S S A D T F L T T A Q W T L V 226
 TACAACAATCGGTTATGGAGCAATGTGATTCTCTCGATGGCGCGGTTGAC 950
 Q Q S V M E Q C D S L D G A V D 242
 GGCGTAATTGAAGCTATCGATCAATGCCACCCGGTCTTTGAGCAACTCAT 1000
 G V I E A I D Q C H P V F E Q L I 259
 CTGCAGACCGGGACAAAATGCTTCGGAATGTCTGACAGGAAAGCAAGTCA 1050
 C R P G Q N A S E C L T G K Q V N 276
 ATACCGCCCAACTCGTCCTTTCCCAATCTACGGAACAAAAGGAGAATTC 1100
 T A Q L V L S P I Y G T K G E F 292
 CTCTATCCGCGCATGCAGCCCGGAGTAGAAAATGTGACATGTACATAAC 1150
 L Y P R M Q P G V E N V D M Y I T 309
 CTACAACGGTGACCCATTTCGCTACAGCACCGACTGGTACAAATACGTCG 1200
Y N G D P F A Y S T D W Y K Y V V 326
 TTTTCAGCGATCCAAATTGGGATCCCGCAACCTTGAACGCGCAGGATTAC 1250
F S D P N W D P A T L N A Q D Y 342
 GAGATTGCGCTTGCTCAAAACCCGTCCAATATCCAGACATTTGAGGGTGA 1300
E I A L A Q N P S N I Q T F E G D 359
 TTTATCCGCTTTCCGCGATGCAGGAGCTAAAGTCTTGACCTATCACGGCA 1350
 L S A F R D A G A K V L T Y H G T 376

CTGCAGACCCGATTATCACGGGGGAGACGTCAAAGGTATACTACCGTCAC	1400
<u>A D P I I T G E T S K V Y Y R H</u>	392
GTGCTGAGACCATGAACGCAGCTCCAGAGGAATTAGATGAGTTTTATCG	1450
V A E T M N A A P E E L D E F Y R	409
CTATTTCCGGATTGGAGGTATGAGCCACTGCGGTGGAGGCACGGGAGCCA	1500
Y F R I G G M S H C G G G T G A T	426
CGGCGATTGGTAATGTGCTCAGTGCGCAATGGAGCAATGATCCTGACGCT	1550
A I G N V L S A Q W S N D P D A	442
AATGTGTTGATGGCGATGGTACGCTGGGTTGAGGAAGGGGTTGCTCCGGA	1600
N V L M A M V R <u>W V E E G V A P E</u>	459
GTATATTCGTGGTGCTTCGCTTGGTAGTGGGCCGGGAGCAAAGGTTGAGT	1650
<u>Y I R</u> <u>G A S L G</u> S G P G A K V E Y	476
ATACTCGGCGGCATTGCAAGTATCCGACGAGGAATGTTTATGTTGGGCCT	1700
T R R H C K Y P T R <u>N V Y V G P</u>	492
GGAAATTGGACGGACGAGAATGCGTGGAATGTATTTGTAGTAGCTTCA	1750
<u>G N W T D E N A W K</u> C I L * *	505

3.4.3. Southern blots

In order to determine how many copies of the *faeC* gene are present in the *Tal. stipitatus* genome, a Southern blot was performed with *EcoRI* restriction enzyme digested genomic DNA. The blot was probed with the *faeC* genomic DNA fragment amplified by PCR using the TsFAEC-N1F/TsFAEC-I3R primer pair (Table 2-3). As the *EcoRI* site appears once in the *faeC* gene sequence, a single copy gene would generate two fragments. Two bands were detected resulting from the two predicted *EcoRI* fragments, as would be the case for a single copy gene (Figure 3-8).

A second Southern blot was performed in order to determine if the *faeC* probe has sufficient homology to hybridise with genomic DNA from other fungal species and therefore identify new potential sources of Type-C feruloyl esterases. Genomic DNAs were digested with *EcoRI* and separated by agarose gel electrophoresis. The TsFAEC coding gene appears to share significant nucleotide identity with hybridising genomic fragments from *Penicillium funiculosum* and *Aspergillus*

oryzae (Figure 3-9). The sequence homologies detected between the *Tal. stipitatus* *faeC* gene and sequences within the *Penicillium* and *Aspergillus* genomes reflect the phylogenetic relationships between these fungal species (Taylor *et al.*, 1990). On the other hand, the two thermophilic fungal species, *Tal. thermophilus* and *Humicola grisea*, did not appear to possess such sequences, since no band could be detected for either on the blot.

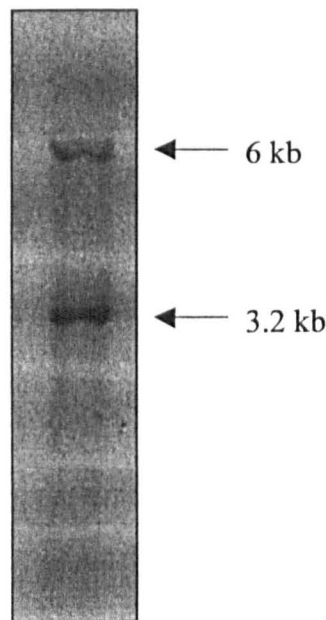


Figure 3-8: Southern blot of *EcoRI* digested *Talaromyces stipitatus* genomic DNA probed with *faeC* genomic fragment. The genomic DNA sequence of the TsFAEC gene contains a single *EcoRI* site and therefore two specific fragments (6 kb and 3.2 kb) were expected from a single copy gene encoding TsFAEC.

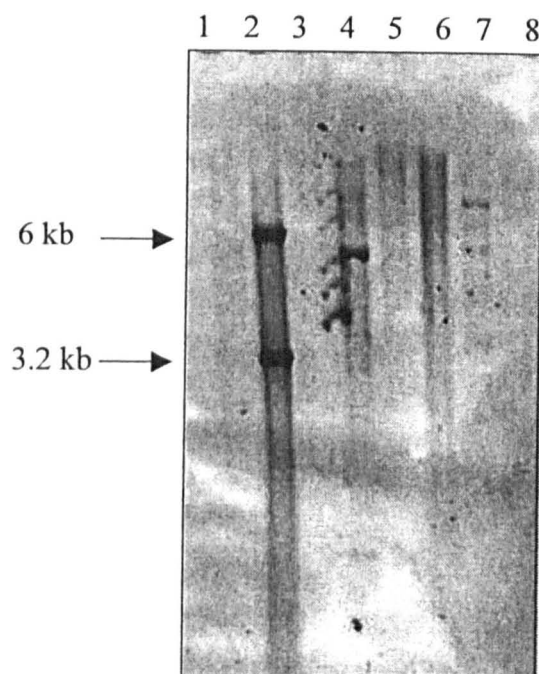


Figure 3-9: Southern blot performed with genomic DNA from various fungal species. Fungal genomic DNAs were digested by the restriction enzyme *Eco*RI and hybridised with *faeC* genomic fragment. 1: *Talaromyces thermophilus*; 2: *Talaromyces stipitatus*; 3: *Pleurotus sajor-caju*; 4: *Penicillium funiculosum*; 5: *Neurospora crassa*; 6: *Humicola grisea*; 7: *Aspergillus oryzae*; 8: *Aspergillus niger*.

3.5. DISCUSSION

Feruloyl esterases have previously been classified into Type-A (e.g. *A. niger* FAEA) and Type-B (e.g. *A. niger* CinnAE (FAEB), *Piromyces equi* CEH, *P. funiculosum* FAEB) enzymes based on their specificity for the aromatic moiety of substrate, their specificity for the primary sugar linkage, and their ability to release ferulic acid dehydrodimers from esterified substrates (de Vries *et al.*, 2002b; Fillingham *et*

al., 1999; Kroon *et al.*, 1999; Kroon *et al.*, 2000). *Tal. stipitatus* grown on WB or SBP is able to produce Type-A (TsFAEA) and Type-B (TsFAEB) feruloyl esterases respectively, with activity profiles similar to those obtained for the characterised esterases of *A. niger*. In addition, we have found *Tal. stipitatus* to produce an esterase that is distinct from the Type-A and Type-B classes of feruloyl esterase. This feruloyl esterase has been designated as a Type-C enzyme (TsFAEC), and is the first example of a non-modular feruloyl esterase that encompasses most of the activities of the Type-A and Type-B esterases, with the exception of the release of diferulates. This is the first report of an organism producing these three distinct forms of feruloyl esterase.

In order to provide a rapid and ready source of the TsFAEC enzyme for industrial use, and to enable further biochemical characterisation, we have cloned the structural gene and cDNA for this enzyme. Recombinant production of the enzyme is reported in Chapter 6 (Expression and characterisation of recombinant TsFAEC in *Pichia pastoris*) of this thesis.

CHAPTER 4. FERULOYL ESTERASE ACTIVITIES OF NEUROSPORA CRASSA

4.1. INTRODUCTION

Neurospora crassa is probably the best studied of all filamentous fungi. There is a wealth of genetic and biochemical information available on this fungus. It is also among the fastest growing of all the filamentous fungi (the doubling time of the mycelial mass under optimal conditions is 2 hours). It is known to grow well on plant cell walls materials, having the ability to secrete hydrolytic enzymes to digest cell wall carbohydrate polymers. The production of enzymes of the cellulase complex by *N. crassa* grown on cellulosic substrates such as wheat straw has been widely investigated (Romero *et al.*, 1999; Yazdi *et al.*, 1990a; Yazdi *et al.*, 1990b) but little is known about the secretion of feruloyl esterases.

As *Neurospora crassa* has been used in our laboratory in diverse studies we decided to investigate the production of feruloyl esterases by this filamentous fungus in parallel with *Tal. stipitatus*.

4.2. FERULOYL ESTERASE ACTIVITIES IN THE CULTURE SUPERNATANT OF NEUROSPORA CRASSA

4.2.1. Detection of feruloyl esterase activities

Neurospora crassa ST A (74 A) was grown in minimal Vogel's medium, supplemented with 2% (w/v) sucrose for 24 hours then transferred on Vogel's 2% (w/v) SBP or 2% WB for 9 days. Culture supernatant samples were removed every 24 hours and assayed for ferulic acid esterase activities against the methyl esters of caffeic, sinapic, ferulic, and *p*-coumaric acids (Chapter 2, section 2.8.1.), using spectrometric methods (Ralet *et al.*, 1994b). Feruloyl esterase activities were detected following growth on SBP and WB (Figure 4-1 and Figure 4-2). The

substrate activity profile however, suggested the presence of more than one type of feruloyl esterase.

Samples removed in the first three days of growth on WB showed feruloyl activity against MFA, MSA, MpCA and not MCA, whereas samples removed during the first three days of growth on SBP showed feruloyl activity against MFA, MCA, MpCA and not MSA. As feruloyl esterases are discriminated based on their ability to utilise MSA and diferulates (de Vries and Visser, 2001; Fillingham *et al.*, 1999; Kroon *et al.*, 1997a; Kroon *et al.*, 1996; Kroon *et al.*, 1999; Kroon *et al.*, 2000), Type-A and Type-B ferulic acid esterase activities could be identified from *N. crassa* grown on WB or SBP. At days 7 - 8 for SBP and 8 - 9 for WB, feruloyl esterase activity was detected against all four synthetic monomeric substrates. This observation was reminiscent of what was observed at day 7 of the *Tal. stipitatus* fermentation on SBP. These data confirm that *N. crassa*, like *Tal. stipitatus* and *A. niger*, is able to induce and adapt the production of ferulic acid esterases according to the carbon source present in the culture medium and probably the degree of degradation of the plant cell wall source. *N. crassa* is therefore a potential source of feruloyl esterases.

In addition to feruloyl esterase activities, culture supernatant samples were assayed for xylanase and pectinase (polygalacturonase) activities (Figure 4-3 and Figure 4-4) (Chapter 2, sections 2.8.2. and 2.8.3.). Xylanase activity was detected following growth on SBP and WB and was stable throughout the growth period with a slight decrease towards the end of the incubation time (days 7 - 9). Interestingly, xylanase activity was also detected after 48 hours of incubation on sucrose minimal medium, suggesting that xylanase and feruloyl esterase expression are not regulated in the same way in presence of sucrose. Similar results have been previously reported for *A. niger* regarding the expression of xylanase and feruloyl esterase activities in presence of glucose in the culture medium (Faulds and Williamson, 1994). Pectinase (polygalacturonase) activity however, was only detected following growth on SBP and WB. The pectinase activity profile was different between the growth substrates with higher activities recorded after growth on SBP but with a delay in the expression when compared to growth on WB.

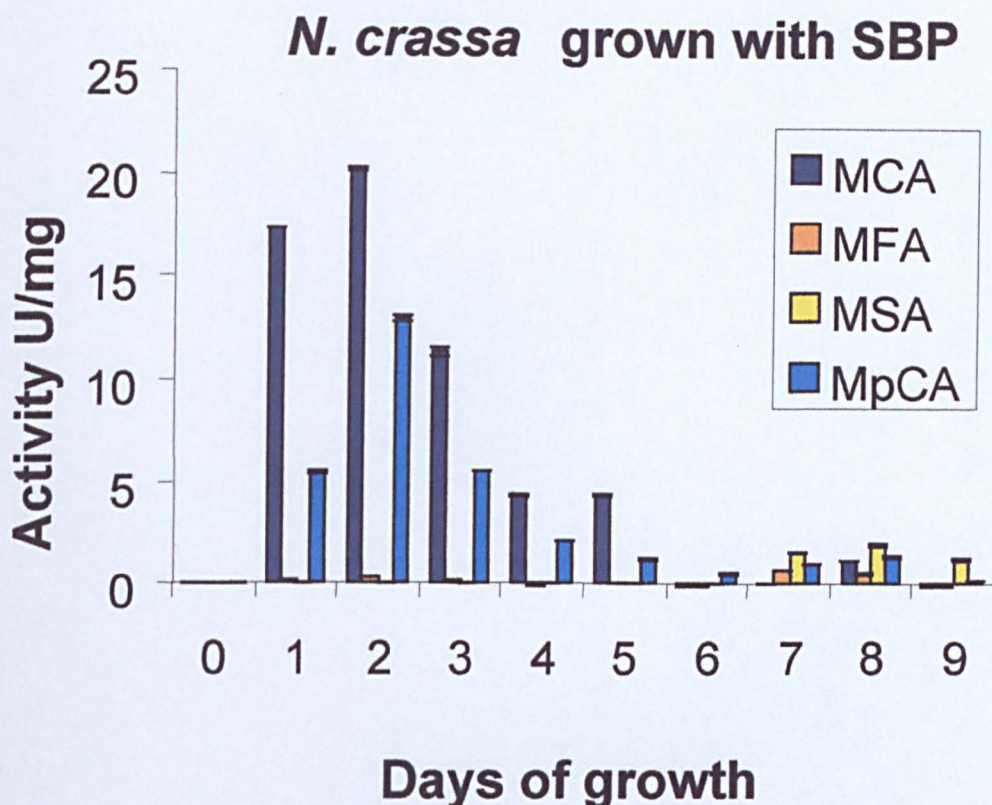


Figure 4-1: Feruloyl esterase activities (U/mg) detected in culture supernatants of *Neurospora crassa* grown with sugar beet pulp. Feruloyl activities have been measured against MCA (dark blue), MFA (orange), MSA (yellow) and MpCA (pale blue), as a function of the incubation time (0-9 days) (Chapter 2, section 2.8.1.). Day 0 corresponds to the supernatant sample analysed after 24 hours of growth with Vogel's plus 2 % sucrose.

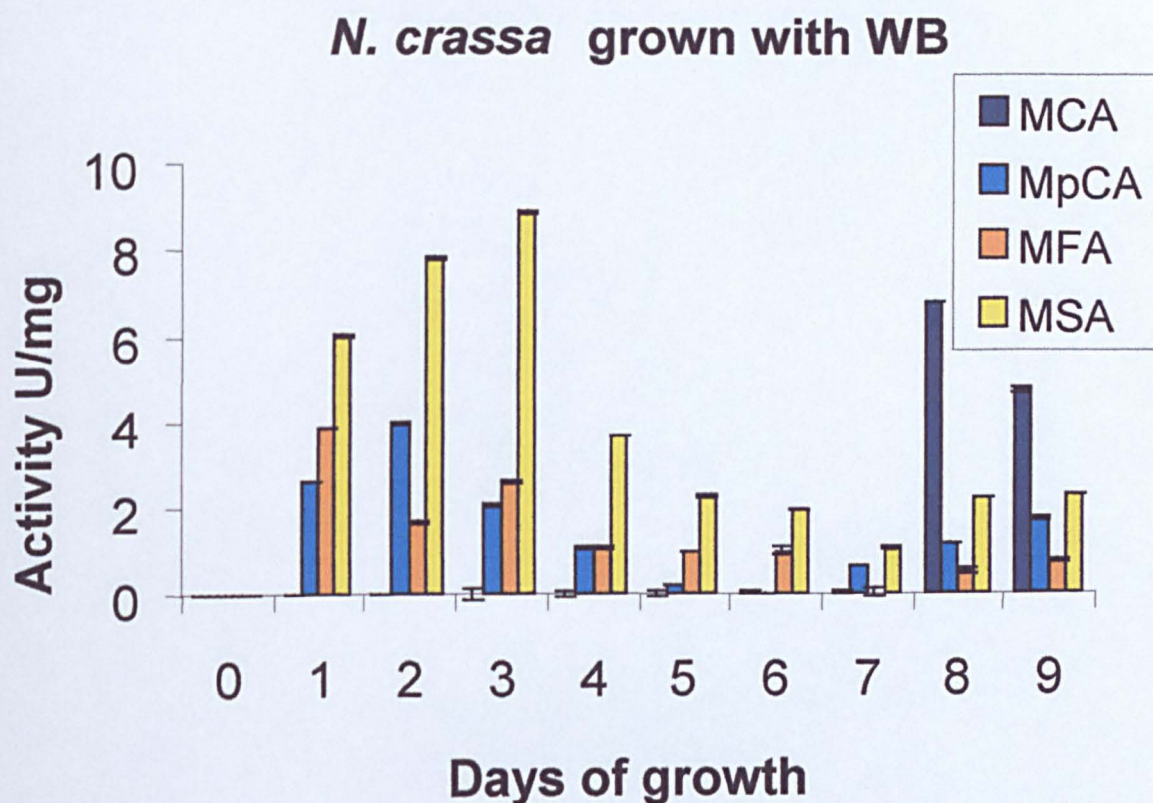


Figure 4-2: Feruloyl esterase activities (U/mg) detected in culture supernatants of *Neurospora crassa* grown with wheat bran. Feruloyl activities have been measured against MCA (dark blue), MFA (orange), MSA (yellow) and MpCA (pale blue), as a function of the incubation time (0-9 days) (Chapter 2, section 2.8.1.). Day 0 corresponds to the supernatant sample analysed after 24 hours of growth with Vogel's plus 2 % sucrose.

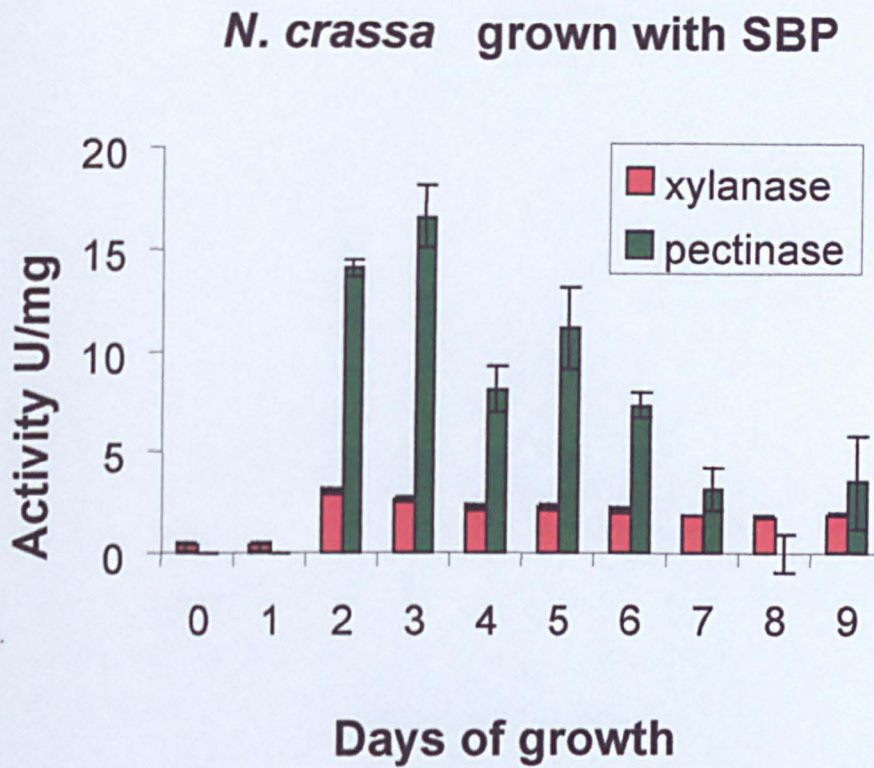


Figure 4-3: Xylanase (in red) and pectinase (polygalacturonase) (in green) activities (U/mg) detected in culture supernatants of *Neurospora crassa* grown with sugar beet pulp as a function of the incubation time (0-9 days) (Chapter 2, sections 2.8.2 and 2.8.3.). Day 0 corresponds to the supernatant sample analysed after 24 hours of growth with Vogel's plus 2 % sucrose.

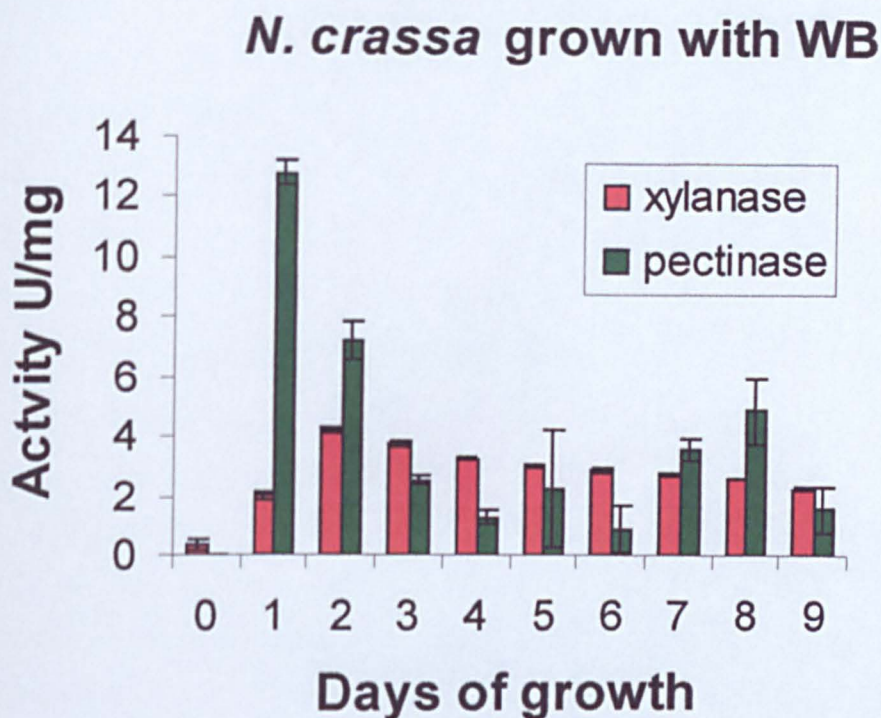


Figure 4-4: Xylanase (in red) and pectinase (polygalacturonase) (in green) activities (U/mg) detected in culture supernatants of *Neurospora crassa* grown with wheat bran as a function of the incubation time (0-9 days) (Chapter 2, sections 2.8.2 and 2.8.3.). Day 0 corresponds to the supernatant sample analysed after 24 hours of growth with Vogel's plus 2 % sucrose.

4.2.2. Identification of putative feruloyl esterase genes

In previous studies, labelled cDNA reverse-transcribed from mRNA extracted from mycelia of *N. crassa* grown on either sucrose or SBP were used to probe duplicate plaque lifts of a genomic library in order to identify genes differentially expressed on SBP (Thomas *et al.*, 1988). The library screen revealed several positives clones. Restriction digests of the cloned DNAs were transferred to Southern blots and hybridised with the labelled cDNA to identify the fragments containing the genes of interest, which were in turn subcloned into the vector pEMBL18 for DNA sequencing.

The feruloyl esterase activity profiles detected in the culture supernatant of *N. crassa* revealed the presence of putative Type-B and C activities following growth on SBP. Putative Type-A and C activities were detected following growth on WB. In order to identify the genes coding these putative Type-B or C feruloyl esterases, we decided to analyse the sequencing data available from the *N. crassa* genomic library screen for genes expressed during growth on SBP. The translation of one of these clones exhibited sequence identity with N-terminal and internal peptide sequences (62 % and 60 %, respectively) of *Tal. stipitatus* FAEB, 50 % identity with a Type-B feruloyl esterase from *P. funiculosum* (Kroon *et al.*, 2000) and 45 % identity with an acetylesterase reported from *Aspergillus awamori* (Koseki *et al.*, 1997). These sequence relationships suggested that the *N. crassa* genomic sequence encodes a Type-B feruloyl esterase (Crepin *et al.*, 2002). Subsequent studies were focused to establish if this gene encoded a functional Type-B feruloyl esterase (chapter 5: Characterisation of recombinant NcFae-1 from *Pichia pastoris*). The *N. crassa* sequence was deposited in the public database and appears as the *fae-1* gene and encodes ferulic acid esterase Fae-1 (GenBank Accession Number: AJ293029).

In addition, a BLAST search performed on the *N. crassa* genomic database (<http://www-genome.wi.mit.edu/>) using various feruloyl esterase sequences available from the NCBI database (nr), revealed five further open reading frames (ORFs) exhibiting significant protein sequence identity. Two ORFs encode putative feruloyl

esterases with 43 - 41 % identity with FAEA from *P. funiculosum* (AJ312296) and XYLD from *Ps. fluorescens* (Ferreira *et al.*, 1993). Three further ORFs encode putative enzymes showing 48 - 45 % identity with Fae-1 from *N. crassa* and 46 - 44 % identity with FAEB from *P. funiculosum* (Kroon *et al.*, 2000). One of these latter ORFs encodes a putative modular enzyme that features a fungal-type cellulose-binding domain (44 % identity with FAEB from *P. funiculosum*). These sequence similarities reflect the diversity and the multiplicity of esterase activities produced by *N. crassa* when grown on plant cell wall materials, and offer a number of genes to investigate in terms of their regulation and protein coding capacities.

4.3. CLONING OF FAE-1 GENE CODING FOR A TYPE-B FERULOYL ESTERASE FROM NEUROSPORA CRASSA

4.3.1. Overlapping-PCR

In order to assess the catalytic function of the corresponding enzyme and provide a rapid and ready source of enzyme it was decided to over-express the *N. crassa* genomic clone (*fae-1*) encoding a putative Type-B feruloyl esterase in a heterologous system. Comparative analysis of the sequence indicated a reading frame of 876 bp was present but interrupted by a single intron, which needed to be removed before protein expression could be attempted (Chapter 2, section 2.4.1.). A cDNA for this gene was therefore constructed by overlapping-PCR from *N. crassa* genomic DNA. Specific primers were designed based on the genomic sequence in order to independently amplify the sequence up-stream (Nc-ssN1F and Nc-I1R primers, Table 2-2) and down-stream (Nc-I1F and Nc-C1R primers, Table 2-2) of the intron, the reverse primer Nc-I1R having a tail with a sequence that matches the forward primer Nc-I1F and vice versa. Two independent PCR amplifications were carried out and the two resulting PCR-fragments were joined as overlapping fragments, filled-in with dNTPs using Klenow enzyme and re-amplified as a single PCR fragment using Nc-ssN1F and Nc-I1R primers (Figure 4-5). After cloning in pCR[®]2.1-TOPO[®], the nucleotide sequence of the overlapping-PCR product was determined.

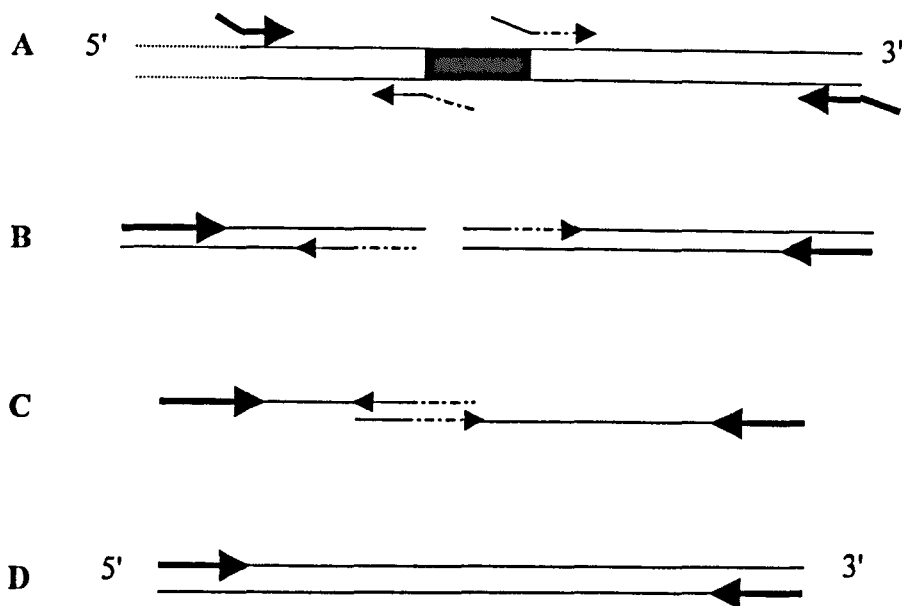


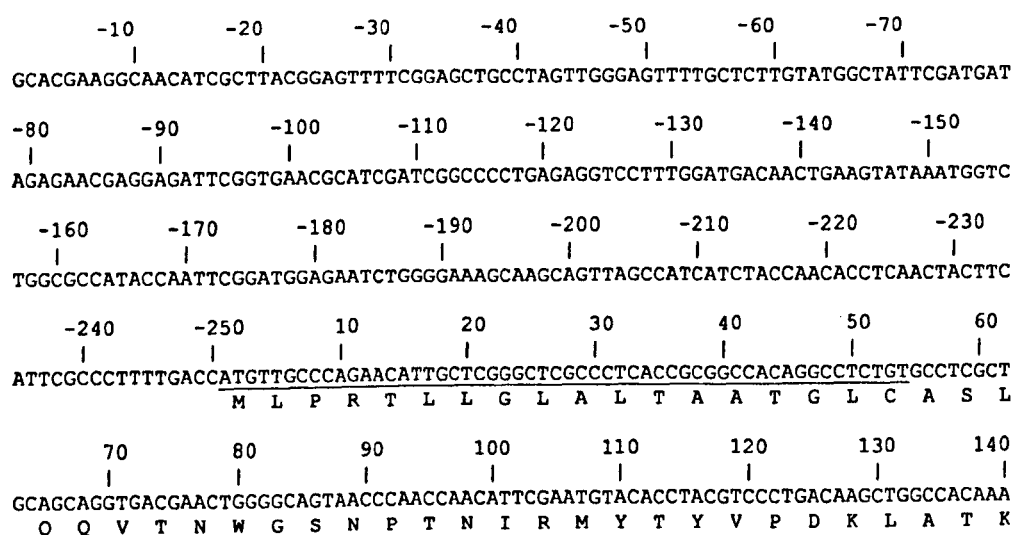
Figure 4-5: Schematic of the overlapping-PCR strategy (Chapter 2, section 2.4.1.). A) The relative positions of the primers designed for the overlapping PCR. The black box represents the intron, and the dotted line the signal sequence. The N-terminal and C-terminal primers are appended with nucleotide sequence to introduce restriction sites: *Bam*H1 and *Nde*1 sites 5' of the N-terminal primer and *Not*1 and *Bam*H1 sites 3' of the C-terminal primer. B) Two independent PCR reactions were carried out to amplify the sequences upstream and downstream of the intron. C) The two PCR fragments were allowed to anneal before filling in. D) The 3'-extremities were synthesised using Klenow enzyme and the entire fragment was re-amplified as a single PCR product.

4.3.2. Sequence analysis

The open reading frame of the *fae-1* gene encodes a protein of 292 amino acids including an 18 amino acids signal peptide predicted by the computer program SignalP (Figure 4-6) (Nielsen *et al.*, 1997). The calculated average mass of the mature protein is 29 286 Da and the theoretical pI is 8.26. Inspection of the protein sequence reveals the presence of two motifs characteristic of the serine esterase

family (G-X-S-X-G) (Brenner, 1988; Dodson and Wlodawer, 1998). The first motif (G-D-S-L-G) is centred on the serine 91 but is not conserved within the *P. funiculosum* cinnamoyl esterase sequence or the acetylcysteine sequence from *Aspergillus awamori* (Koseki *et al.*, 1997; Kroon *et al.*, 2000). The second motif (G-T-S-S-G) centred on serine 118 is conserved between Fae-1, the *P. funiculosum* cinnamoyl esterase and the *Aspergillus awamori* acetylcysteine sequences as well as other members of the serine esterase family (Kroon *et al.*, 2000). Based on the conservation of the sequence environment around these putative active sites serine residues, the active site of the enzyme is predicted to be centred at serine 118 (G-T-S-S-G). The translated sequence of Fae-1 does not feature a recognizable cellulose-binding domain, which is in contrast to the Type-B ferulic acid esterase reported from *P. funiculosum* (Kroon *et al.*, 2000).

Figure 4-6: Nucleotide and deduced amino acid sequences of the *fae-1* gene from *Neurospora crassa*. The signal peptide is underlined. The 56 bp intron is shown in lower case. Two sites containing the consensus motif (G-X-S-X-G) for the active-site serine are shown in grey boxes with the putative active-serines in bold. Putative N-linked glycosylation sites (occurring at the Asn residue within the Asn-X-Thr/Ser/Cys- sequence, where X can be any residue except proline) are shown in reverse field. An asterisk indicates the stop codon.



150 160 170 180 190 200 210
| | | | | | |
GCCCGCCATTATTGTGGCTgtgagtgcgcttctcttgattctcacacatcggcgatgctaacgaggcaatgatagCTC
P A I I V A L

220 230 240 250 260 270 280 290
| | | | | | |
CACGGCTGTGGCGGCACCGCCCTTCCTGGTACTCGGGCACACGCCTACCCTCCTACGCCGACCAATACGGCTTCATC
H G C G G T A P S W Y S G T R L P S Y A D Q Y G F I

300 310 320 330 340 350 360 370
| | | | | | |
CTCATCTATCCCGGCACGCCAACATGTCCAACCTGCTGGGGAGTTAACGACCCTGCTTCTCTCACGCACGGAGCCGGC
L I Y P G T P N M S N C W G V N D P A S L T H G A G

380 390 400 410 420 430 440 450
| | | | | | |
GGCGACTCCCTCGGTATCGTTGCCATGGTGAACACCATCGCCAAATACAACGCCGACGCTTCGCGCGTCTATGTC
G D S L G I V A M V N Y T I A K Y N A D A S R V Y V

460 470 480 490 500 510 520 530
| | | | | | |
ATGGGCACCTCCTCGGGCGGCATGATGACCAACGTAATGGCCGCCACTTATCCGGAGGTCTTTGAGGCCGGGGCTGCG
M G T S S G G M M T N V M A A T Y P E V F E A G A A

540 550 560 570 580 590 600
| | | | | | |
TACTCGGGCGTTGCGCACGCTTGTTCGCCGGTGCTGCGTCTGCTACTCCCTTTTCGCCCACACAGACCTGCGCCAGG
Y S G V A H A C F A G A A S A T P F S P N Q T C A R

610 620 630 640 650 660 670 680
| | | | | | |
GGACTGCAGCACACTCCGGAGGAATGGGGCAACTTTGTGAGGAATTCTTACCGGGGTACAGGGACGTAGACCAAGG
G L Q H T P E E W G N F V R N S Y P G Y T G R R P R

690 700 710 720 730 740 750 760
| | | | | | |
ATGCAGATCTACCATGGCTCGCCGACAATCTGGTATATCCAAGGTGTGCGATGGAGGCCCTGAAGCAGTGGTCAAC
M Q I Y H G L A D N L V Y P R C A M E A L K Q W S N

770 780 790 800 810 820 830 840
| | | | | | |
GTGCTGGGGGTAGAATTTTCGAGGAATGTCAGCGGAGTGCCGAGCCAGGCGTATACGCAGATTGTGTATGGAGATGGC
V L G V E F S R N V S G V P S Q A Y T Q I V Y G D G

850 860 870 880 890 900 910 920
| | | | | | |
AGCAAGCTGGTTGGGTATATGGGTGCAGGCGTGGGGCATGTGGCACCACGAATGAGCAGGTAATGTTGAAGTTCTTT
S K L V G Y M G A G V G H V A P T N E Q V M L K F F

930 940 950 960 970 980 990
| | | | | | |
GGGTTGATCAACTAGATGTCGAGCAAAGGTTAGTTGATGGCTTGATGAAGGAATGCGGACGGGAATGAT
G L I N *

4.3.3. Northern and Southern blots

To confirm the differential expression of the putative feruloyl esterase gene, RNAs were extracted after growth on either sugar beet pulp, wheat bran or sucrose and analysed by Northern blot (Figure 4-7). Two transcripts specific to the growth on SBP were detected by hybridisation with the genomic DNA probe (*fae-1*).

Expression on SBP and absence on WB and sucrose is a profile consistent with the expression of Type-B feruloyl esterase gene observed from other fungal species (de Vries and Visser, 2001; de Vries *et al.*, 2002b; Kroon *et al.*, 1996; Kroon *et al.*, 2000).

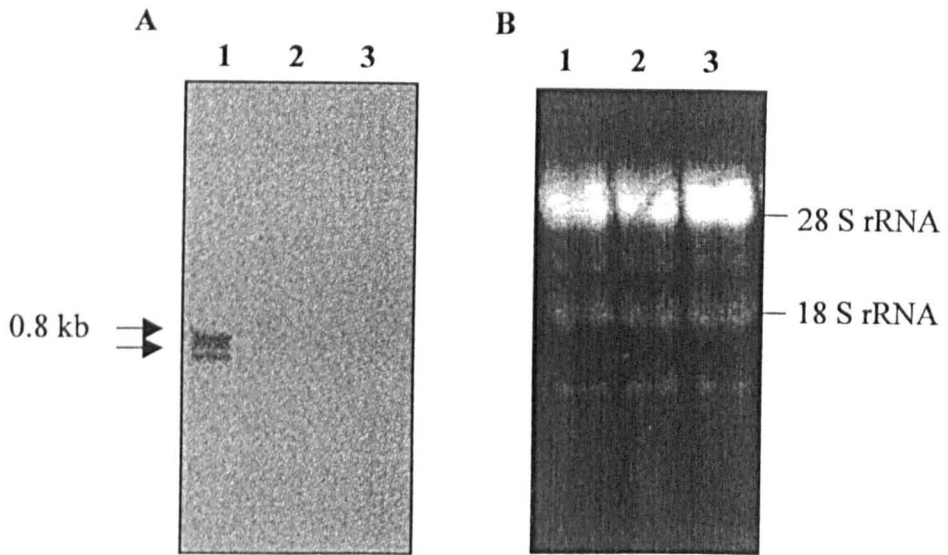


Figure 4-7: A) Northern blot of RNAs extracted from *N. crassa* following 6 hours of growth on pre-digested selective media: SBP, lane 1; WB, lane 2; following 48 hours of growth on minimal Vogel's 2% sucrose, lane 3. The membrane was hybridised with the *N. crassa* genomic clone coding for a Type-B ferulic acid esterase. B) Ethidium bromide stain of the membrane showing equal loading of the RNAs.

In order to determine whether the two transcripts observed arise from two independent genes or are products of a single gene, a Southern blot of genomic DNA was performed. *N. crassa* genomic DNA was digested with *EcoRI* (known to cut once in the genomic sequence) and hybridised with the genomic sequence to produce two bands as might be anticipated for a single copy gene (Figure 4-8). The transcripts are therefore likely products of the same gene. In parallel, a Southern blot was

performed with genomic DNA extracted from several fungi in order to determine if other fungal species contain sequences homologous to the *N. crassa* *fae-1* gene sequence. Hybridising fragments were observed with the genomic DNAs of *P. funiculosum*, *Tal. stipitatus*, *Tal. thermophilus*, *A. oryzae* and *A. niger* (Figure 4-9), suggesting all these fungi have the potential to produce a feruloyl esterase similar to Fae-1 (Crepin *et al.*, 2002). Interestingly, genomic DNA of the thermophilic strain *Tal. thermophilus* hybridises with the *N. crassa* genomic sequence. This would suggest that although attempts to isolate feruloyl esterases from the culture supernatant of *Tal. thermophilus* grown on sugar beet pulp were unsuccessful, the fungus does possess the genetic information necessary for feruloyl esterase production.

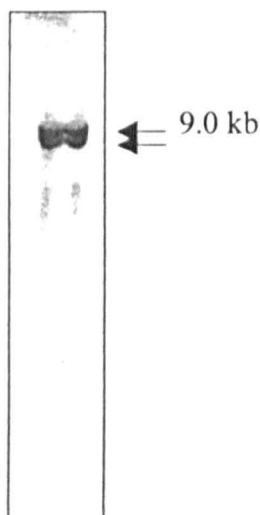


Figure 4-8: Southern blot of *N. crassa* genomic DNA digested with the restriction enzyme *EcoRI*. The relative molecular sizes of the main hybridisation products are recorded alongside the blot.

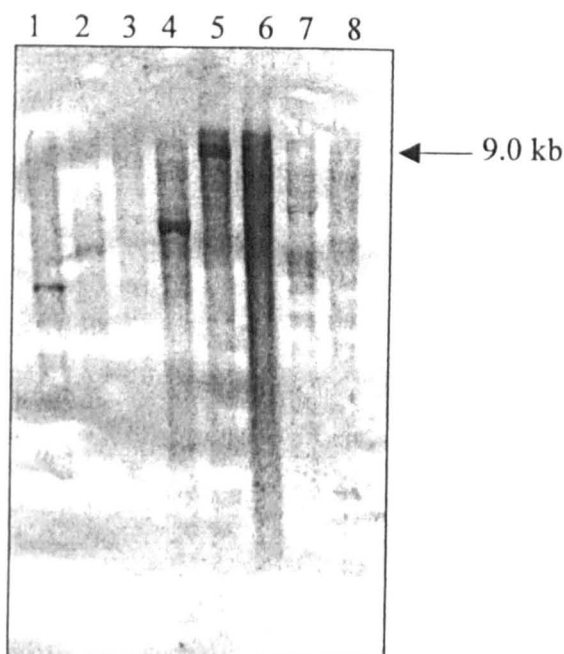


Figure 4-9: Fungal genomic DNAs digested with *EcoRI* and hybridised with the *N. crassa fae-1* genomic clone. 1: *Talaromyces thermophilus*, 2: *Talaromyces stipitatus*, 3: *Pleurotus sajor-caju*, 4: *Penicillium funiculosum*, 5: *Neurospora crassa*, 6: *Humicola grisea*, 7: *Aspergillus oryzae*, 8: *Aspergillus niger*.

The mRNA expression of the putative feruloyl esterase gene was also analysed by Northern blot at 48 hours intervals over the period of fermentation with raw particulate SBP and WB substrates (Figure 4-9). The putative Type-B feruloyl esterase mRNA was expressed during the early stages of growth on SBP but expression decreased later in the fermentation. The *fae-1* mRNA was expressed at parallel time points to the Type-B enzyme activity recorded in Figure 4-1. The *fae-1* mRNA expression was barely detectable throughout the course of the fermentation with WB (Figure 4-2).

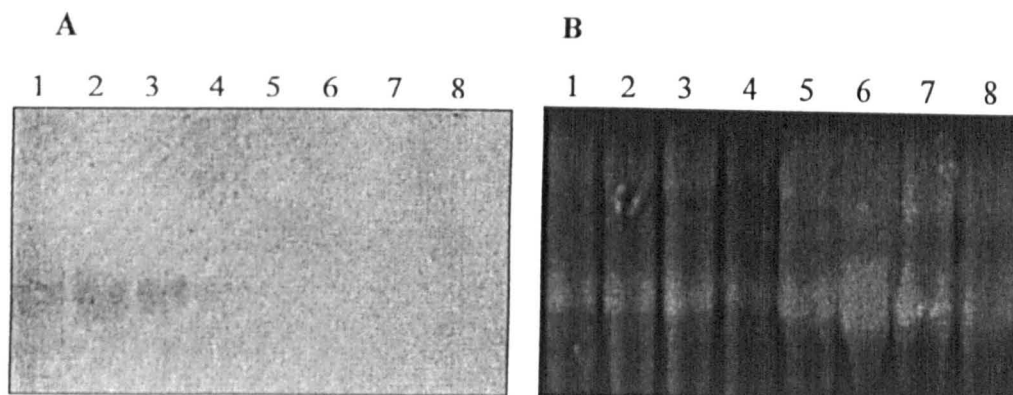


Figure 4-10: A) Northern blot of RNAs extracted from *N. crassa* following two, four, six and eight days of fermentation with 2% SBP, lanes 1-4; and 2% WB, lanes 5-8. B) Ethidium bromide stain of the membrane showing equal loading of the RNAs.

4.4. DISCUSSION

N. crassa was shown to produce either Type-A or Type-B feruloyl esterase activities when grown on either wheat bran or sugar beet pulp respectively. The substrate utilisation profiles are similar to those reported for the *A. niger* and *Tal. stipitatus* esterases against synthetic methyl esters when cultured on similar carbon sources (de Vries and Visser, 2001; de Vries *et al.*, 2002b; Faulds and Williamson, 1994; Kroon *et al.*, 1996). During the early stages of the growth period (days 2 - 5), *N. crassa* produces Type-A and Type-B carbon source-dependent esterase activities but later in the fermentation of either wheat bran or sugar beet pulp (days 5 - 6) these activities become repressed to be replaced by the expression of a new activity (days 7 - 9), characterised by its ability to hydrolyse all four synthetic substrates tested (MCA, MFA, MSA, MpCA). This later activity may represent a compound activity of replacement Type-A and B enzymes, alternatively the activities may be the product

of a single enzyme with an hitherto unrecognised ability to deal with broad substrate structures, similar to that reported for the Type-C feruloyl esterase from *Tal. stipitatus*. The induction of an enzyme with broad substrate activity could replace the specialised enzymes to complete the digestion of complex plant materials. The *fae-1* gene is only strongly expressed during the early stages of the fermentation with SBP, and may only contribute residual activity at later stages. The *fae-1* gene is weakly expressed throughout the fermentation on WB. Therefore the feruloyl Type-B enzyme substrate utilisation observed at the later stages of growth on SBP and WB may arise due to the expression of an alternative gene or genes.

The presence of *N. crassa* genomic sequences showing strong identities with other reported feruloyl esterases is consistent with the idea that *N. crassa* is capable of producing more than one type of feruloyl esterase, and our activity measurements, as well as the time-course mRNA expression analysis, would suggest that these act as either specialist or generalist enzymes dependent on the substrate and its degree of depolymerisation. It is therefore very likely that most fungi can produce more than one type of ferulic acid esterase and utilise a battery of these enzymes to assist in the degradation of cell wall materials.

In order to confirm the function of the putative *fae-1* gene product and provide a ready source of enzyme for study, the *N. crassa* cDNA was over-expressed in *Pichia pastoris* (Chapter 5: Characterisation of recombinant NcFae-1 from *Pichia pastoris*).

CHAPTER 5. CHARACTERISATION OF RECOMBINANT NCFAE-1 FROM *PICHIA* *PASTORIS*

5.1. INTRODUCTION

Many plant cell walls contain phenolic acid residues that are ester-linked to the polysaccharide network. In grasses these phenolic compounds are mainly found esterified to arabinoxylans (5-*O*-feruloyl group). In dicotyledons, such as spinach and sugar beet, ferulic acid is esterified to the *O*-2 or *O*-3 position of arabinose and to the *O*-6 position of galactose residues in pectin (Fry, 1982; Ralet *et al.*, 1994c). Feruloyl esterases are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in the plant cell wall. These enzymes have been classified as either Types-A or B dependent on their substrate specificity for aromatic moieties. The enzymes show a preference for the phenolic linkage to the primary sugar and vary in their ability to release dehydrodiferulic acids from esterified substrates. Regarding the specificity against synthetic substrates, Type-A feruloyl esterases are active against methyl ferulate, methyl sinapate, methyl *p*-coumarate and not methyl caffeate whereas Type-B are active against methyl caffeate, methyl ferulate, methyl *p*-coumarate and not methyl sinapate. Only Type-A ferulic acid esterases are able to hydrolyse synthetic ferulate dehydrodimers and to release dimers from agro-industrial materials as they have preference for more hydrophobic substrates with bulky substituents on the benzene ring (Kroon *et al.*, 1997a; Kroon *et al.*, 1999; Williamson *et al.*, 1998a).

The two major ferulic acid esterases of *Aspergillus niger* have been the focus of several studies over the last decade. However, in the recent years, our knowledge of the ferulic acid esterase family has expanded with reports of new enzyme activities, the characterisation of gene sequences and the first crystal structure (Prates *et al.*, 2001; Schubot *et al.*, 2001). Molecular analysis of ferulic acid esterase genes and their predicted protein sequences has revealed that many of these enzymes are

modular, comprising of a catalytic domain translationally fused to a non-catalytic cellulose-binding domain (Coutinho and Henrissat, 1999; Ferreira *et al.*, 1993; Fillingham *et al.*, 1999; Kroon *et al.*, 2000) or are produced as monomeric enzyme units pre-designated to be assembled into longer cohesive units, such as the cellulosome of *Clostridium* (Shoham *et al.*, 1999).

The ability of the filamentous fungus *Neurospora crassa* to produce alternative ferulic acid esterase activities in response to the availability of plant cell wall carbohydrates has been investigated resulting in the identification of a gene (*fae-1*) specifically induced on sugar beet pulp (Chapter 4, section 4.2). The translation of this gene was analysed and compared to other sequences available in the database. Sequence identity (50 %) with PfFAEB as well as the expression profile of the *N. crassa* gene strongly suggests that *fae-1* encodes Type-B feruloyl esterase.

5.1.1. The *Escherichia coli* expression system

In order to confirm the catalytic function of the putative *N. crassa fae-1* product attempts were initiated to express the protein in a heterologous system. Initially the *E. coli* pET system was employed as a quick route to express Fae-1 recombinant protein. A cDNA of the *fae-1* gene, excluding its native signal peptide sequence, was cloned in the expression vector pET3a and transformed into *E. coli* BL21 (DE3)/pLysS cells (Chapter 2, sections 2.5.1. and 2.6.1.). Various temperatures and inducing IPTG concentrations were tested to find the best conditions in order to produce the largest quantity of soluble protein. Although *E. coli* produced the recombinant protein it proved to be insoluble, more than likely concentrated in inclusion bodies and non-active irrespective of the conditions employed (Figure 5-1) (Crepin *et al.*, 2002).

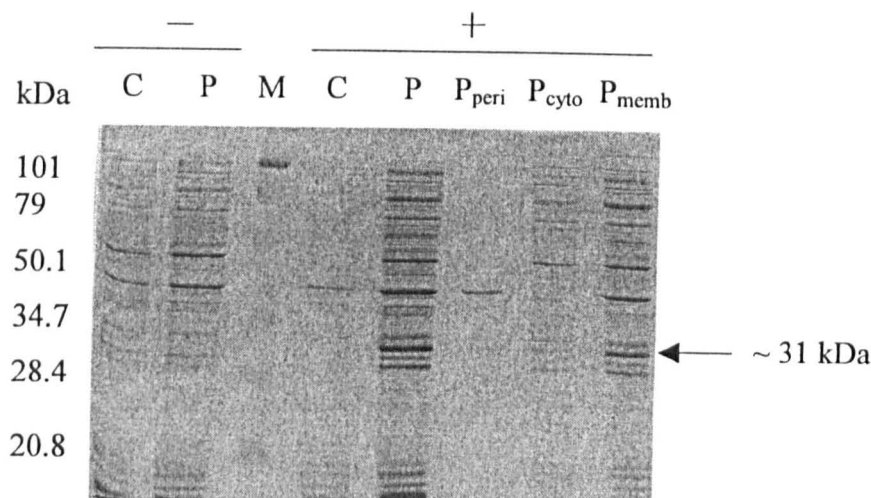


Figure 5-1: Expression of *N. crassa* Fae-1 in *E. coli* (Chapter 2, section 2.6.1.). Cells were grown at 37 °C and induced at OD₆₀₀= 0.4 with 0.4 mM IPTG. C: control strain transformed with pET3a. P: protein expression strain transformed with pET3a/*fae-1*. P_{peri}: periplasmic fraction, P_{cyto}: cytoplasmic fraction, P_{memb}: membrane and inclusion bodies fraction. M: standard protein molecular weight. -: pre-induction. +: post-induction.

Rather than trying to solubilise the recombinant protein and its recovery after refolding the enzyme from denaturing solutions, we decided to try the *Pichia pastoris* yeast expression system. It had already been reported that the recovery of active PfFAEB from inclusion bodies was poor (Kroon *et al.*, 2000).

5.1.2. The *Pichia pastoris* expression system

The *Pichia pastoris* expression system was selected as it had already proven to efficiently secrete an active feruloyl esterase from a fungal source (Juge *et al.*, 2001). Being a eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic

expression systems such as protein processing, protein folding, and post-transcriptional modifications like glycosylation. A clear advantage to the use of the *P. pastoris* expression system is the high yield of secreted recombinant protein against a background of very limited host protein secretion, which allows for rapid purification of the target protein.

5.1.2.1. *The Pichia pastoris alcohol oxidase promoter*

Pichia pastoris is a methylotrophic yeast, capable of metabolising methanol as sole carbon source. The first step in the methanol metabolism pathway is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. Alcohol oxidase has a poor affinity for O₂, and *Pichia pastoris* compensates by producing large quantities of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *P. pastoris*. However, there are two genes in *P. pastoris* coding for alcohol oxidase: *AOX1* and *AOX2*. The *AOX1* gene is responsible for the majority of alcohol oxidase activity in the cell. Expression of the *AOX1* gene is tightly regulated at the transcription level and induced in presence of methanol to a very high level, reaching approximately 30 % of the total soluble protein in cells grown with methanol as sole carbon source (Ellis *et al.*, 1985). While *AOX2* is 97 % identical in amino acid sequence to *AOX1*, the expression of the gene is much lower in presence of methanol leading to a poor growth of *Pichia pastoris* on methanol (Mut^S phenotype: methanol utilisation sensitive phenotype) when only *AOX2* alcohol oxidase is available (Cregg *et al.*, 1989; Koutz *et al.*, 1989). Therefore, based on the properties of the inducible *AOX1* promoter, an expression system has been developed in *P. pastoris*.

5.1.2.2. *Pichia pastoris expression vectors*

Pichia pastoris strain GS115 carries an auxotrophic mutation in the gene for histidinol dehydrogenase (*his4*) that prevents synthesis of histidine, which must therefore be added to the growth media. The *HIS4* gene is present in *Pichia* expression vectors and allows the selection of transformants (phenotype His⁺) by their ability to grow on histidine-deficient medium. Induction of heterologous protein

expression is driven specifically by methanol, acting at the *AOX1* promoter (P_{AOX1}) present in *Pichia* expression vectors. These vectors are designed to be linearised with a restriction enzyme such that His⁺ recombinants are generated by recombination at the *AOX1* or *his4* loci (Figure 5-2). Linear DNA can generate stable transformants of *P. pastoris* via homologous recombination between the transforming DNA and regions of homology within the *Pichia* genome (Cregg *et al.*, 1985; Cregg *et al.*, 1989).

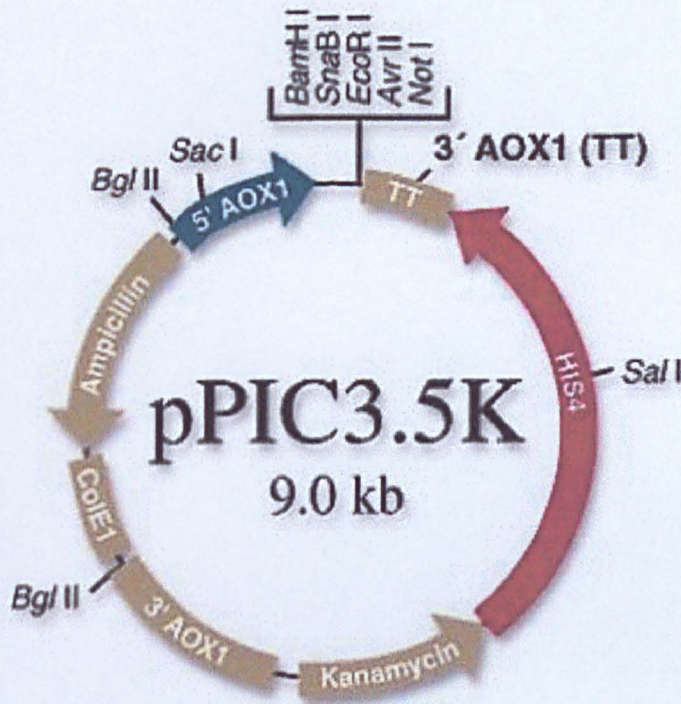


Figure 5-2: The pPIC3.5K expression vector contains the methanol-inducible promoter from *Pichia*, the *AOX1* transcription termination, the *HIS4* *Pichia* gene coding for histidinol dehydrogenase used to complement *Pichia pastoris* GS115 *his4* strains and select recombinant strains, the ampicillin resistance gene allowing selection of *E. coli* transformants and the kanamycin resistance gene which confers resistance to G418 in *Pichia* and kanamycin resistance in *E. coli* (Invitrogen).

5.1.2.3. Recombination and integration events in GS115 *Pichia pastoris*5.1.2.3.1. Gene insertion at *AOX1* or *his4* loci in GS115 *Pichia pastoris*

Gene insertion events can arise from a single crossover event between the *AOX1* or *his4* loci and any of the *AOX1* or *his4* regions on the vector respectively (Figure 5-3), by linearising the recombinant vector at a restriction enzyme site located in *AOX1* regions (*SacI*) or *HIS4* gene (*SalI*) (Figure 5-2). The resulting phenotype of such transformant is His⁺ Mut⁺ (methanol utilisation phenotype), as the *AOX1* gene remains intact. Gene insertion events can also occur between region of homology with non-linearised plasmid or re-ligated plasmid and the *AOX1* or *his4* loci of *Pichia* genome, although at a lower frequency.

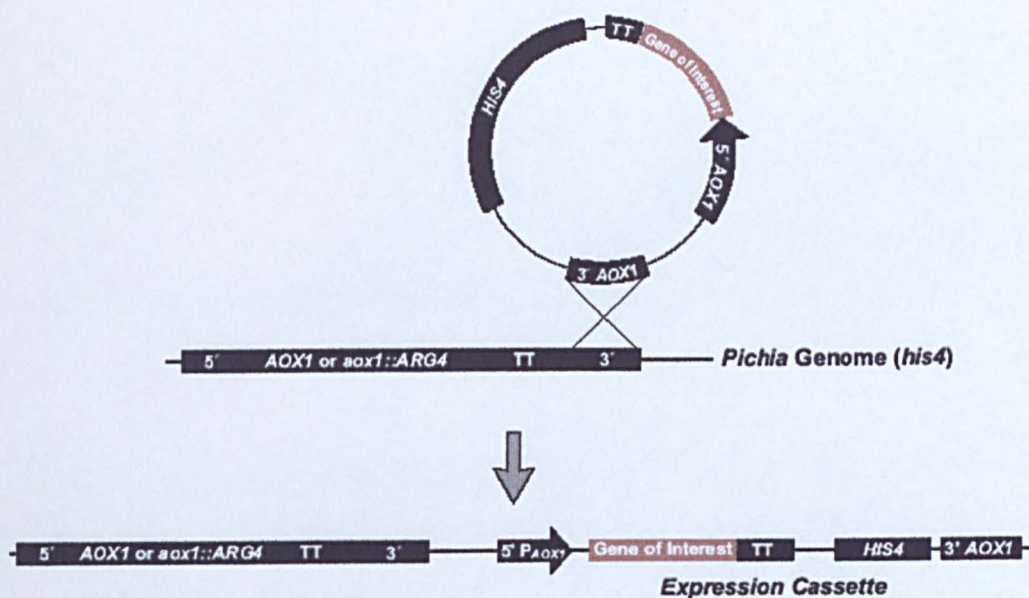


Figure 5-3: Gene insertion at *AOX1*. Gene insertion arises from a single crossover event between the *AOX1* loci and any of the *AOX1* regions on the vector. The resulting phenotype will be His⁺ Mut⁺ (Invitrogen).

5.1.2.3.2. Gene replacement at *AOX1* locus in GS115 *Pichia pastoris*

A gene replacement event arises from a double crossover event between the *AOX1* promoter and 3'*AOX1* regions of the vector and the *Pichia* genome (Figure 5-4), by linearising the recombinant vector at *Bgl*III (or *Dra*I) restriction site (Figure 5-2). This results in the complete removal of the *AOX1* genomic coding region, which is replaced with the expression cassette containing the *AOX1* promoter region (P_{AOX1}), the target gene to be expressed, and the *HIS4* gene, which allows transformant selection. The resulting phenotype of the strain is His⁺ Mut^S due to the loss of alcohol oxidase activity encoded by *AOX1* gene. Mut^S recombinant strains often demonstrate more efficient expression of heterologous protein, as competition for transcription factors of the *AOX1* promoter is eliminated following the presence of a single *AOX1* promoter copy in *Pichia* genome.

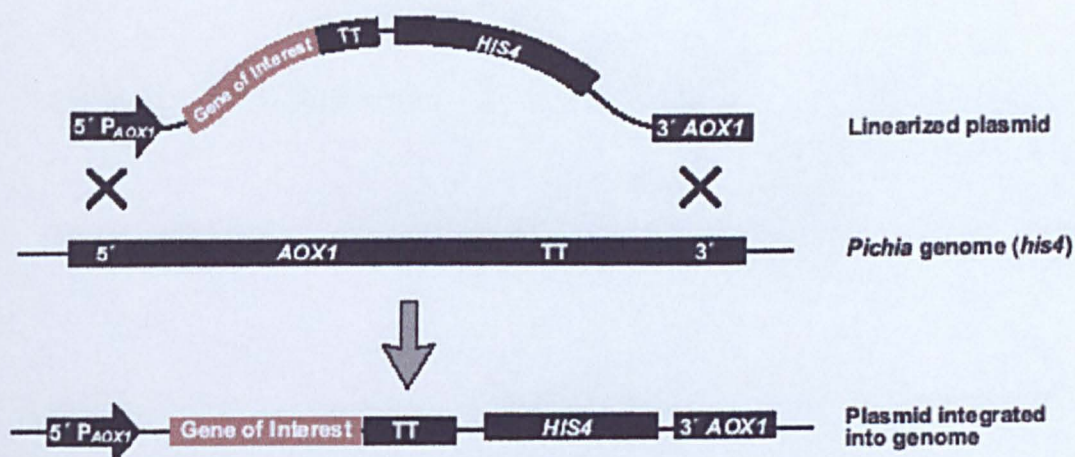


Figure 5-4: Gene replacement at *AOX1*. A double crossover event between the *AOX1* promoter and 3'*AOX1* regions of the vector and the genome results in the replacement of the *AOX1* genomic coding region by the expression cassette (P_{AOX1} , the gene of interest, and *HIS4* gene). The resulting phenotype is His⁺ Mut^S (Invitrogen).

In addition, multiple gene insertion events at a single locus can occur spontaneously with a low but detectable frequency, between 1 and 10 % of all selected His⁺ transformants (Figure 5-5). The initial integration event can be gene insertion or gene replacement and will determine the methanol utilisation phenotype of the transformant.

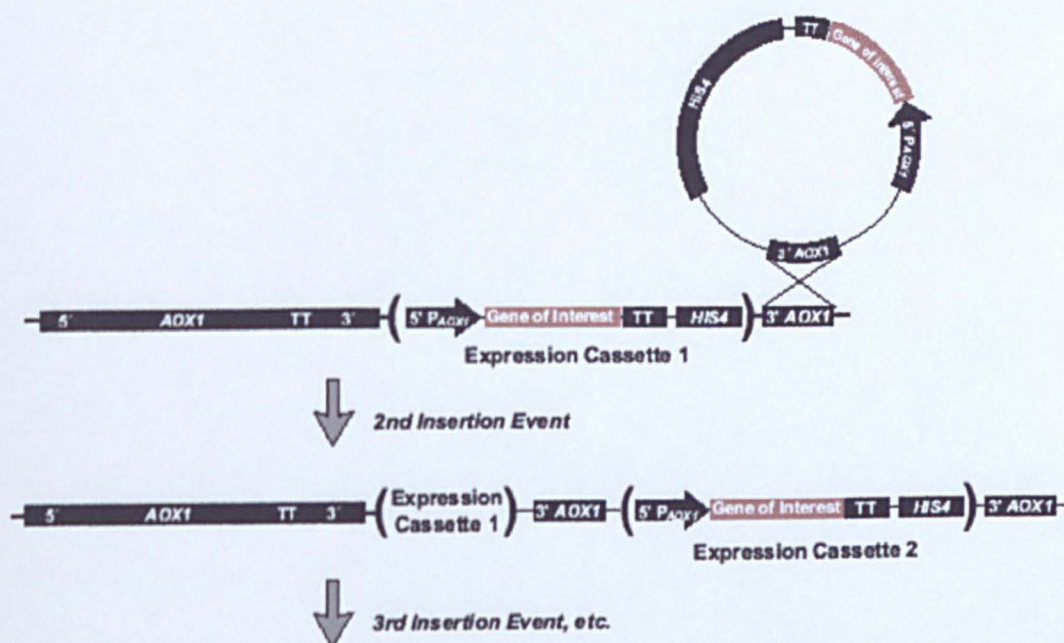


Figure 5-5: Multiple gene insertion. Multi-copy events can occur as gene insertions either at the *AOX1* or *his4* loci (Invitrogen).

5.2. OVER-EXPRESSION OF FAE-1 IN PICHIA PASTORIS

5.2.1. Sub-cloning into the expression vector

The cDNA of the *fae-1* gene from *N. crassa*, containing the native signal sequence, was isolated from the pCR[®]2.1-TOPO[®] cloning vector by restriction endonuclease digestion with *Bam*HI-*Not*I and ligated into the *Bam*HI-*Not*I digested pPIC3.5K expression vector under the control of the alcohol oxidase 1 promoter (*AOX1*) (Figure 5-2) (Chapter 2, section 2.5.2.1.). The resulting expression plasmid (pPIC3.5K/*fae-1*) and the parent vector could be linearised with *Dra*I restriction enzyme, to enable gene replacement at *AOX1* locus of *Pichia pastoris* GS115 (*his4*).

5.2.2. Transformation into *Pichia pastoris*

Recombinant and parental vectors (pPIC3.5K/*fae-1* and pPIC3.5K) were linearised with *Dra*I restriction enzyme and transformed into *Pichia pastoris* GS115 strain using the electroporation method. The efficiency of *P. pastoris* transformation by the electroporation method was in the range of 10^3 to 10^4 colonies per microgram of DNA. His⁺ transformants were selected by their ability to grow on histidine-deficient regeneration medium.

The transformations generated hundreds of His⁺ transformants, which were then scored for their ability to grow on minimal methanol media. To assess the His⁺ transformants for their methanol utilisation phenotype (Mut), colonies were picked and patched onto minimal methanol (MM) and minimal dextrose (MD) plates in a regular pattern. Normal growth on MD plate and slow growth on MM plates were taken as indicative of the Mut^S phenotype, expected to be generated following transformation with *Dra*I linearised DNA targeting gene replacement at the *AOX1* locus. Out of 208 His⁺ transformants screened for replacement of the *AOX1* gene, 13 were of the Mut^S phenotype (Figure 5-6).

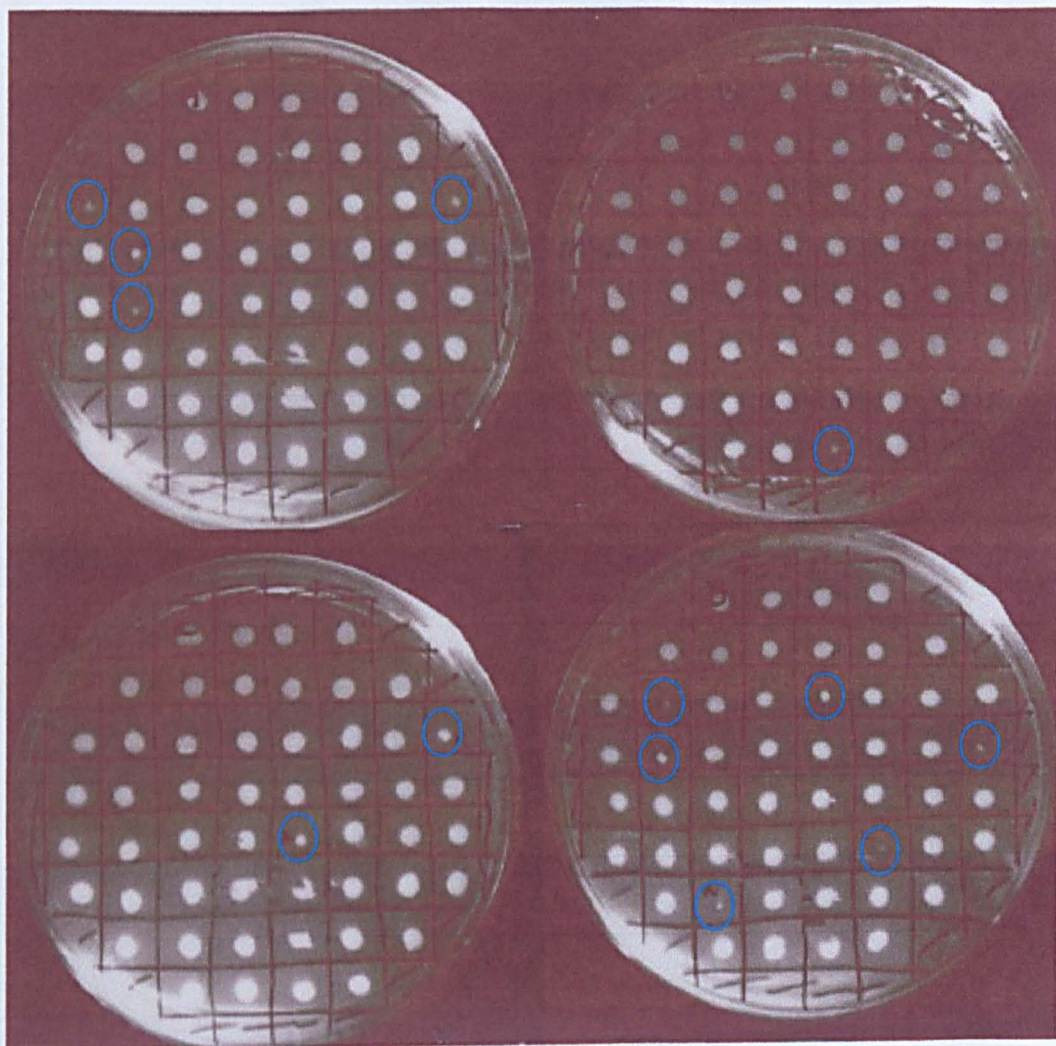


Figure 5-6: His⁺ transformants are assessed for methanol utilisation phenotype on MM plates. Mut^S transformants are encircled in blue.

5.2.3. Expression and purification of the recombinant Fae-1

All thirteen His⁺ Mut^S pPIC3.5K/*fae-1* transformants were screened for recombinant Fae-1 expression in small-scale cultures along with two colonies transformed with the parental vector as a control of the background protein secretion levels. Culture supernatants were analysed by SDS-PAGE for secreted protein products. Five pPIC3.5K/*fae-1* transformants produced a major secreted protein band of approximately 35 kDa. No protein was detected with the vector controls (Figure 5-7).

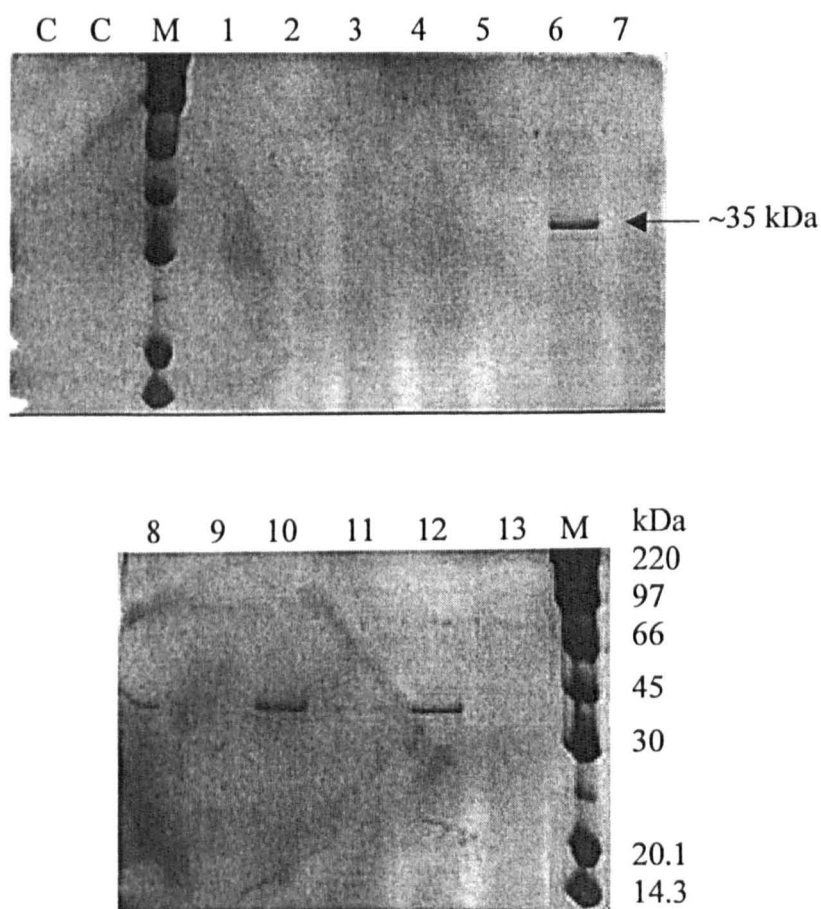


Figure 5-7: SDS-PAGE to analyse the expression of Fae-1 in *P. pastoris* small-scale cultures. M: standard protein molecular weight in kDa; C: vector control; lane 1-13: His⁺ Mut^S pPIC3.5K/*fae-1* transformant clones 1 to 13.

To confirm these cultures were feruloyl esterase-producing transformants, culture supernatants were assayed for activity against MCA, MFA and MSA, using spectrophotometric and HPLC methods. The recombinant protein was found to be active as a feruloyl esterase and shows the characteristics of a Type-B ferulic acid esterase, in that it was inactive against MSA. We therefore have named the *N. crassa* protein Fae-1 as the product of the *fae-1* gene (GenBank Accession Number: AJ293029). The clone expressing the highest enzyme activity (clone 10) was retained for large-scale expression in order to purify and characterise the recombinant enzyme.

The recombinant Fae-1 was transferred by electoblotting to a PVDF membrane for protein sequence analysis. The first 10 amino acids of the N-terminus of the expressed protein were determined: ASLQQVTNWG. The sequence is identical to the sequence predicted for the mature Fae-1 product, indicating *P. pastoris* was able to efficiently process the *N. crassa* native signal sequence. Four potential N-linked glycosylation sites can be predicted from the Fae-1 amino acid sequence (Figure 4-6). Glycosylation of the *P. pastoris* product was confirmed by ESI-MS which would explain the mass difference of 6 kDa between the calculated average mass of the mature protein and that estimated from SDS-PAGE for the recombinant enzyme. Proteins secreted by *P. pastoris* have a majority of N-linked glycosylation of the high-mannose type. N-type glycosylation occurs on the nitrogen atom of asparagine (Asn) side chains that are part of the sequence -Asn-Xaa-Ser/Thr/Cys- (Xaa can be any amino acid residue except proline) (Kornfeld and Kornfeld, 1985). On average, the length of oligosaccharide chains added post-translationally is 8 - 14 mannose residues, with no terminal α -1,3 glycan linkages, which are believed to be of hyper-antigenic nature (Cregg *et al.*, 1993; Grinna and Tschopp, 1989). Six main peaks were apparent from the mass spectrometry ranging between 34 716 and 35 523 Da (Figure 5-8). The main mass is calculated to be 35 040 Da. The other peaks correspond to different levels of glycosylation of the enzyme, where each peak corresponds to the addition of one sugar unit (162 kDa per hexose group such as fructose, galactose, glucose or mannose). The un-glycosylated protein has a calculated molecular mass of 29 286 Da, thus the main product can be estimated to contain 32 hexose groups, which are likely to be mannose residues.

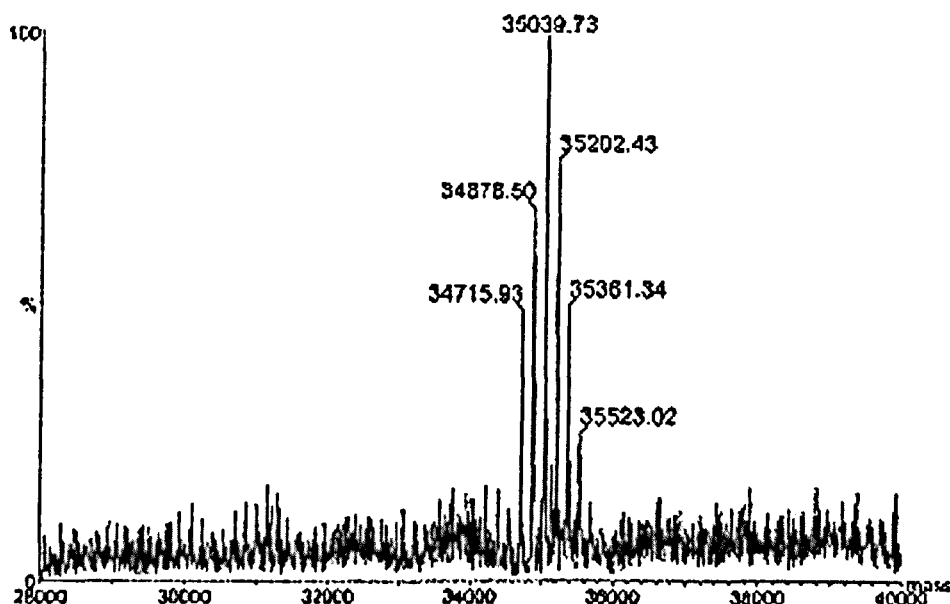


Figure 5-8: Electrospray ionisation mass spectrum of the recombinant *N. crassa* Fae-1 (performed by Dr F. Mellon from the Institute of Food Research, Norwich).

5.2.4. Purification of the recombinant Fae-1

The pPIC3.5K/*fae-1* *Pichia* transformant (clone 10) was grown for 5 days in BMMY media at 30 °C before harvesting (Figure 5-9). Thirty millilitre aliquots of the culture supernatant were fractionated on a butyl sepharose fast flow 4 hydrophobic interaction chromatographic column. A single peak of activity against MCA was eluted (Figure 5-10), and the active fractions were pooled. The enzyme yield after purification was determined to be 73 %. Protein concentration of the recombinant Fae-1 was calculated from the absorbance of the desalted sample at 280 nm. The extinction coefficient of Fae-1 ($51790 \text{ M}^{-1} \text{ cm}^{-1}$) was calculated from the amino acid sequence and the concentration of the purified esterase determined to be 0.048 mg

protein per ml of sample. The yield of secreted recombinant Fae-1 protein from methanol-induced shake-flask culture was 210 mg L⁻¹ after 5 days (Table 5-1).

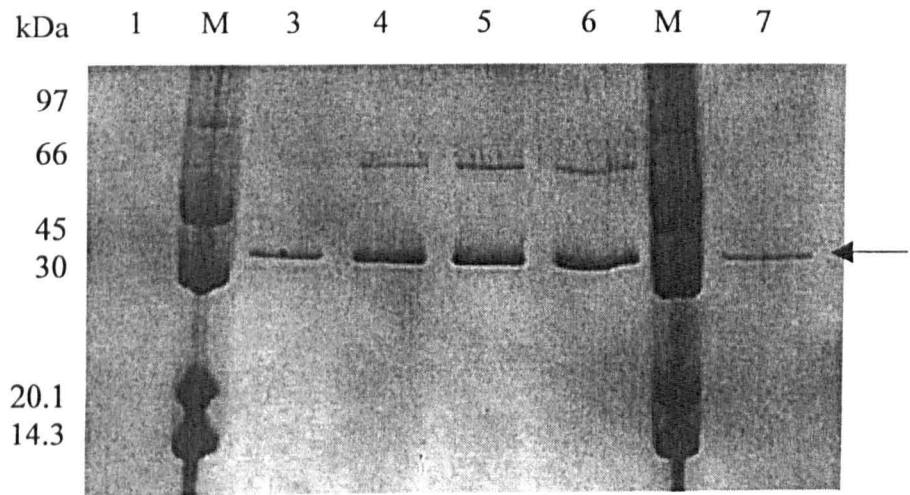


Figure 5-9: SDS-PAGE 12 % acrylamide. M: standard protein molecular weight in kDa. Lane 1: negative vector control. Lanes 3-6: samples of culture supernatant removed daily (days 2-5) from a large-scale culture of *P. pastoris* transformant expressing the recombinant Fae-1 (clone 10). Lane 7: recombinant Fae-1 following purification.

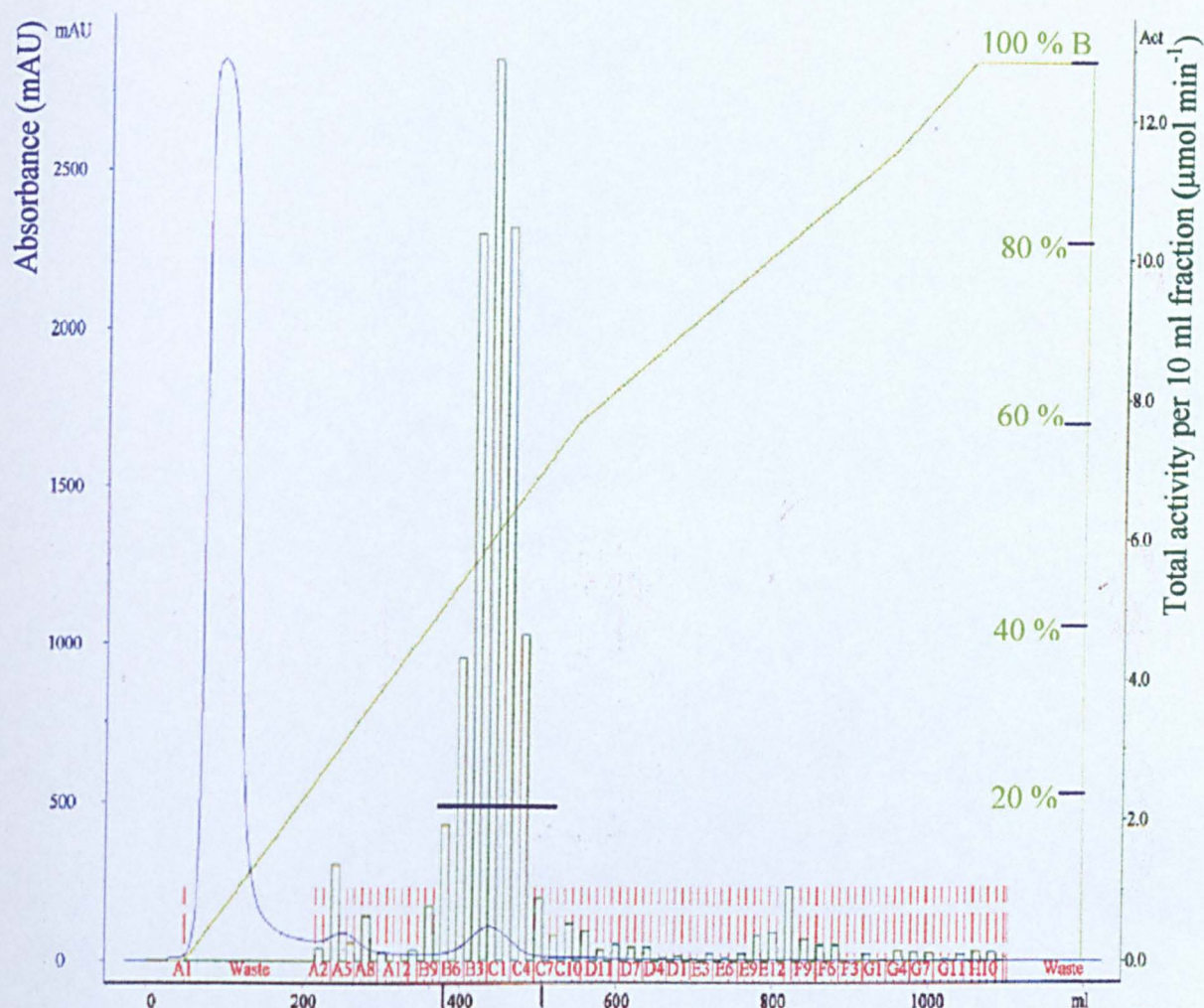


Figure 5-10: Elution profile of the recombinant *N. crassa* Fae-1 on the butyl sepharose fast flow 4 HIC column. The fractions active against MCA were pooled and are marked with a horizontal line, eluting around 400 ml.

Table 5-1: Purification of the recombinant *N. crassa* Fae-1 secreted by *P. pastoris*.

Recombinant enzyme	Activity prior to purification ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Activity after purification ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Yield (%)	Protein after purification ($\mu\text{g ml}^{-1}$)	Total protein for 100 % recovery (mg L^{-1})
Fae-1	2.627 (196 ml)	0.606 (620 ml)	73	48	210

5.2.5. Characterisation of the recombinant Fae-1

Table 5-2 presents the kinetic constants (V_{max} , k_{cat} , K_{m} , $k_{\text{cat}}/K_{\text{m}}$) calculated from initial rates of the recombinant *N. crassa* Fae-1 against hydroxycinnamic acid esters. Substrate concentrations between 0.02 mM and 0.4 mM, were employed to estimate the kinetic constants from the Michaelis-Menten equation (Equation 5-1).

$$\text{Equation 5-1: } v = (V_{\text{max}} \times [\text{S}]) / (K_{\text{m}} + [\text{S}])$$

As expected of a Type-B feruloyl esterase, Fae-1 showed a high catalytic efficiency for the hydrolysis of the methyl esters of caffeate and *p*-coumarate and no activity was detected against methyl sinapate. Methyl caffeate and methyl *p*-coumarate substrates were turned over at relatively high rate, with a preference for methyl *p*-coumarate, but the catalytic efficiency against methyl ferulate was lower. The low rate recorded for the Ara₂F substrate is consistent with a requirement for a longer feruloylated oligosaccharide to produce optimal hydrolysis. The catalytic efficiency of the *N. crassa* Fae-1 to hydrolyse Ara₂F ($12.87 \text{ mM}^{-1} \text{ s}^{-1}$) is, however, 112-fold greater than that recorded for the *A. niger* cinnamic acid esterase CinnAE or the *P. equi* cinnamoyl ester hydrolase CEH (EstA') but 40-fold lower than the catalytic efficiency of *P. funiculosum* FAEB ($530 \text{ mM}^{-1} \text{ sec}^{-1}$) (Fillingham *et al.*, 1999; Kroon *et al.*, 1997b; Kroon and Williamson, 1996; Kroon *et al.*, 2000).

Fae-1 was also tested for its ability to release ferulic acid and *p*-coumaric acid from cell wall material. Fae-1 could be shown to release ferulic acid from spent brewer's grain at a similar level to that reported for the Type-A from *A. niger* (Bartolome *et al.*, 1997b), and to be more efficient at releasing ferulic acid (2.5 % FA released) from sugar beet pulp than *A. niger* Type-B feruloyl esterase (0.88 % FA released) (Kroon and Williamson, 1996), (Table 5-3). Fae-1, however, did not release ferulated dimers from any of the agro-industrial materials tested, which is consistent with the properties of other Type-B feruloyl esterases (Kroon *et al.*, 1996; Kroon *et al.*, 2000). Fae-1 was able to release small amount of *p*-coumaric acid from wheat bran and spent brewer's grain, which is the first report of feruloyl esterase capable of releasing *p*-coumaric acid from un-treated cereal-derived material. The acetyl esterase activity of Fae-1 was investigated using α -naphthyl acetate as substrate. Fae-1 is able to efficiently hydrolyse α -naphthyl acetate with an activity in excess of that measured for FAEA from *A. niger* but lower than that recorded for true acetylxyln esterases (Table 5-2). In addition there are probably constraints on the access of the feruloyl esterases as compared with xylan-esterases as neither FAEA nor Fae-1 can release acetate from cereal cell wall material.

Table 5-2: Substrate specificity of *Neurospora crassa* Fae-1 against synthetic substrates. *Nd: not detected. †Assays performed by Dr. C.B. Faulds at the Institute of Food Research, Norwich.

Synthetic substrates	V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1} \text{s}^{-1}$)
Methyl esters				
MFA	8.97	5.24	0.25	21
MpCA	20.87	12.19	0.021	580
MCA	8.20	4.80	0.048	100
MSA	Nd*	Nd	Nd	Nd
Feryloylated oligosaccharides†				
Ara ₂ F (1-2Ara)	10.34	6.04	0.46	12.87
Acetylated substrates†				
α -Naphthyl acetate	6.65 (NcFae-1)	3.44 (NcFae-1)	1.13 (NcFae-1)	3 (NcFae-1)
	11.64 (AnFAEA)	5.86 (AnFAEA)	5.45 (AnFAEA)	1 (AnFAEA)
Chlorogenated substrates†				
Chorogenic acid	9.14	5.34	0.18	29

Table 5-3: Substrate specificity of *Neurospora crassa* Fae-1 against natural substrates. *Nd: not detected. †Assays performed by Dr. C.B. Faulds at the Institute of Food Research, Norwich. ‡Expressed as % of total alkali-extractable phenolic acids, after 3 hours of incubation at 37 °C with 100 mU of Fae-1.

Natural substrates†	Released of Ferulic Acid‡	Released of <i>p</i> -Coumaric Acid‡	Release of dimers‡
Wheat bran	1.2	0.7	Nd
Spent grain	2	0.7	Nd
Sugar beet pulp	2.5	0	Nd

The pH optimum of the recombinant Fae-1 was determined against MFA at a range of pH values between 3.0 and 8.0 (Figure 5-11). Maximum activity was recorded at pH 6.0 with the enzyme activity stable between pH 6.0 and pH 7.5. The enzyme was generally compromised at acidic pH.

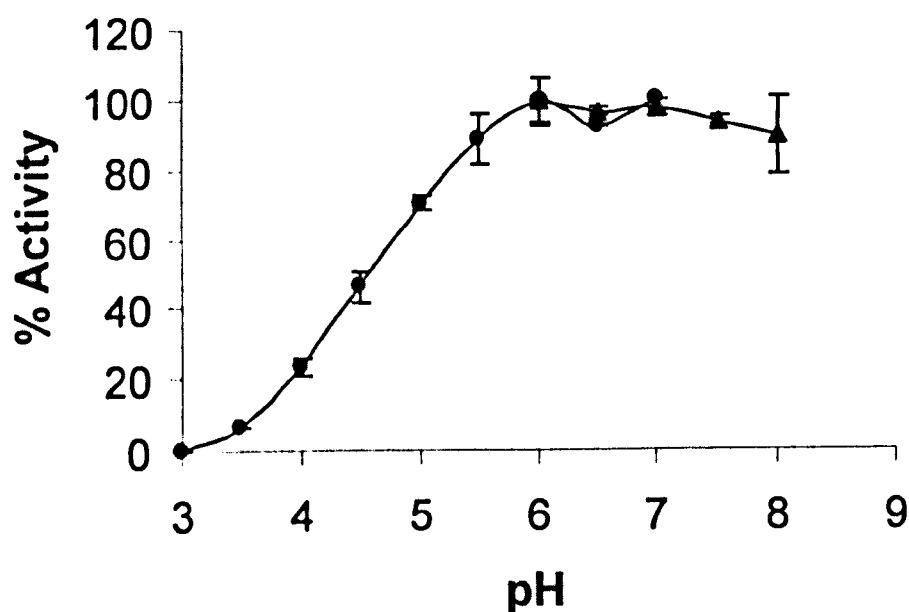


Figure 5-11: The pH activity profiles of the recombinant Fae-1 (Chapter 2, section 2.7.6.). Activities were determined against MFA (0.1 mM) over the range pH 3.0 – 7.0 using Mc Ilvaine's buffer (●) and in the range pH 6.0 – 8.0 using a phosphate buffer (▲).

The temperature optimum of the recombinant Fae-1 was determined against MFA at the range of temperatures between 20 and 80 °C (Figure 5-12). The recombinant enzyme had a temperature optimum of 55 °C for the hydrolysis of MFA. Above this temperature the activity dropped significantly to reach a plateau (24 % activity) between 70 and 80 °C.

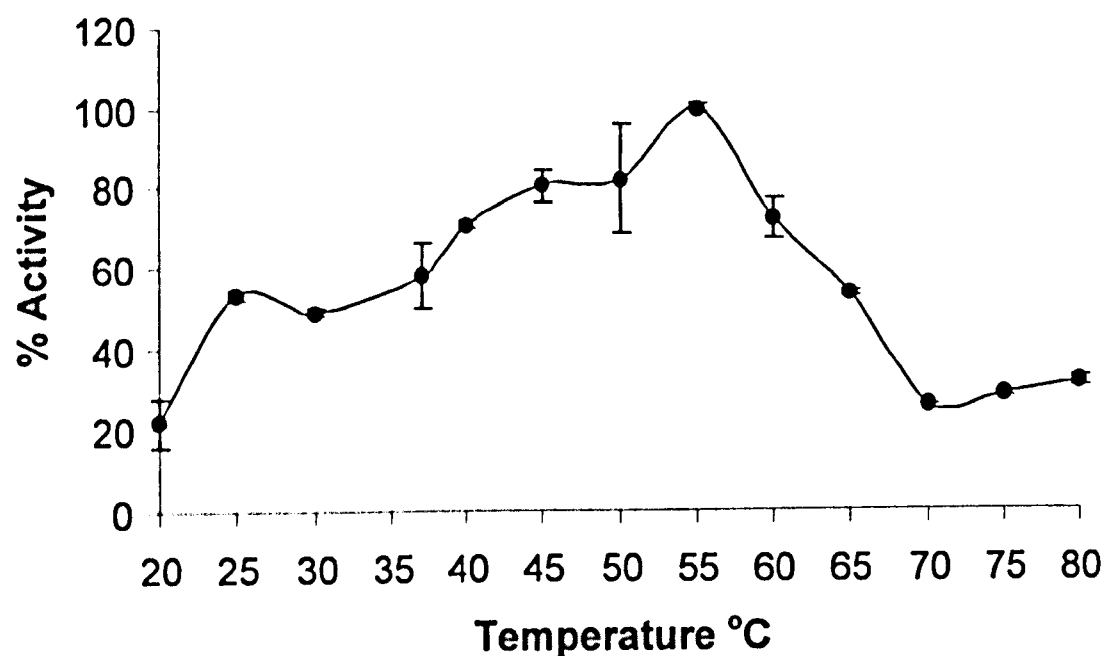


Figure 5-12: The temperature profile of the recombinant Fae-1 determined against MFA (0.1 mM), between 20 - 80 °C (5 °C increment) (Chapter 2, section 2.7.6.).

The temperature stability of the recombinant Fae-1 activity was measured by incubating the enzyme at 60 °C for 60 minutes. Samples were removed every 10 minutes and assayed for residual activity against MFA at 37 °C (Figure 5-13). After 10 minutes at 60 °C the enzyme lost 80% of its activity. However, the residual activity was retained even after 60 minutes at 60 °C.

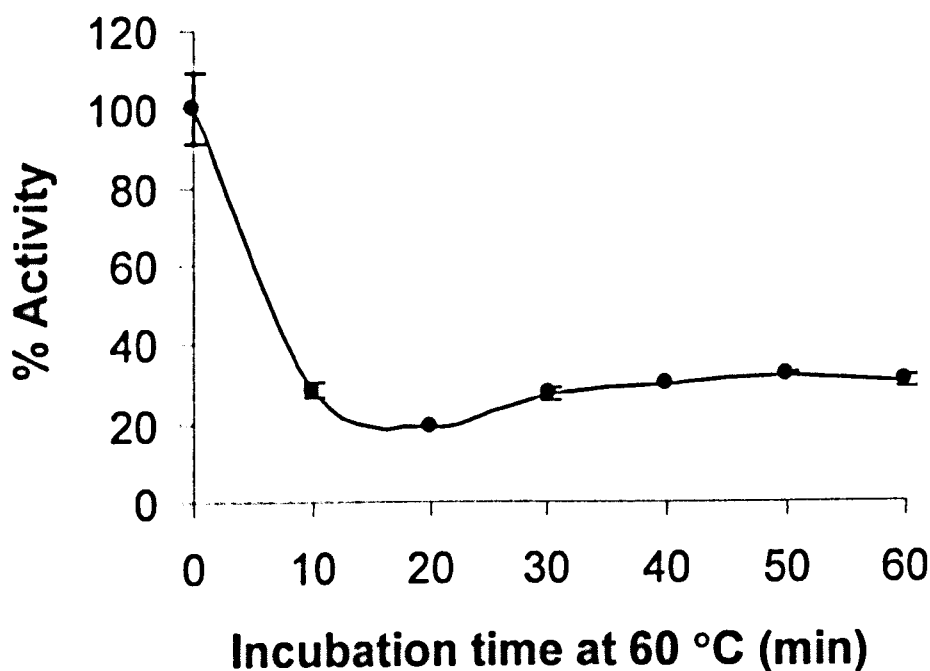


Figure 5-13: The temperature stability of the recombinant Fae-1 (Chapter 2, section 2.7.6.). The temperature stability was estimated at 60 °C over 60 minutes. Samples were removed every 10 minutes and assayed for residual activity at 37 °C against MFA (0.1 mM).

5.2.5.1. Substrate inhibition of Fae-1

At high concentrations of the synthetic monomeric substrates (MCA, MFA, MSA and MpCA) the enzyme activity of Fae-1 was shown to fall. The initial rate data could be related to the rate equation derived for substrate inhibition (Equation 5-2).

$$\text{Equation 5-2:} \quad v = (V_{\max} \times [S]) / (K_m + [S] + ([S]^2/K_i))$$

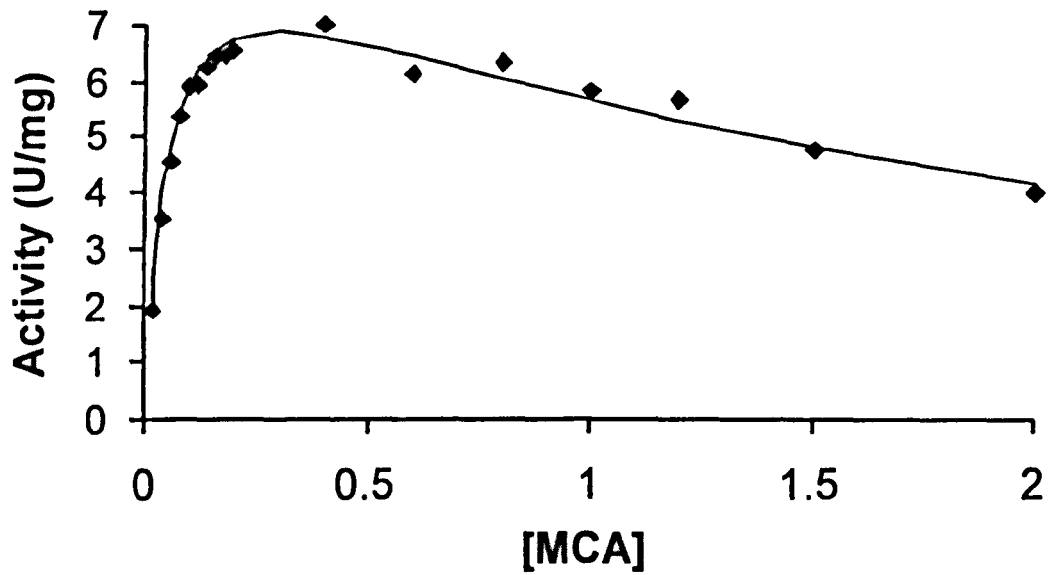
This equation was applied to first estimate the kinetic constants V_{\max} and K_m and to determine K_i values at substrate concentrations from 0.02 to 0.3 mM. As the K_i was large compared with K_m , at low values of substrate (between 0.02 and 0.3 mM) the enzyme rates approximated to the Michaelis-Menten equation. Therefore, at low substrate concentrations the kinetic constants V_{\max} and K_m were determined in the usual way from the Michaelis-Menten equation (Equation 5-1), using the Lineweaver-Burk double-reciprocal plots. The kinetic curves obtained with concentration of MCA from 0.02 to 0.3 mM are shown in Figure 5-14 A and Figure 5-14 B and kinetic constants determined for all four synthetic substrates are presented in Table 5-4.

Table 5-4: Fae-1 kinetic constants determined against synthetic methyl esters.

Substrate	K_m (mM)	K_i (mM)	χ^2
Methyl esters			
MFA	0.25	2.8	0.067
MpCA	0.021	2	0.082
MCA	0.048	2.5	0.003
MSA	Nd*	Nd	Nd

*Nd: not detected.

A



B

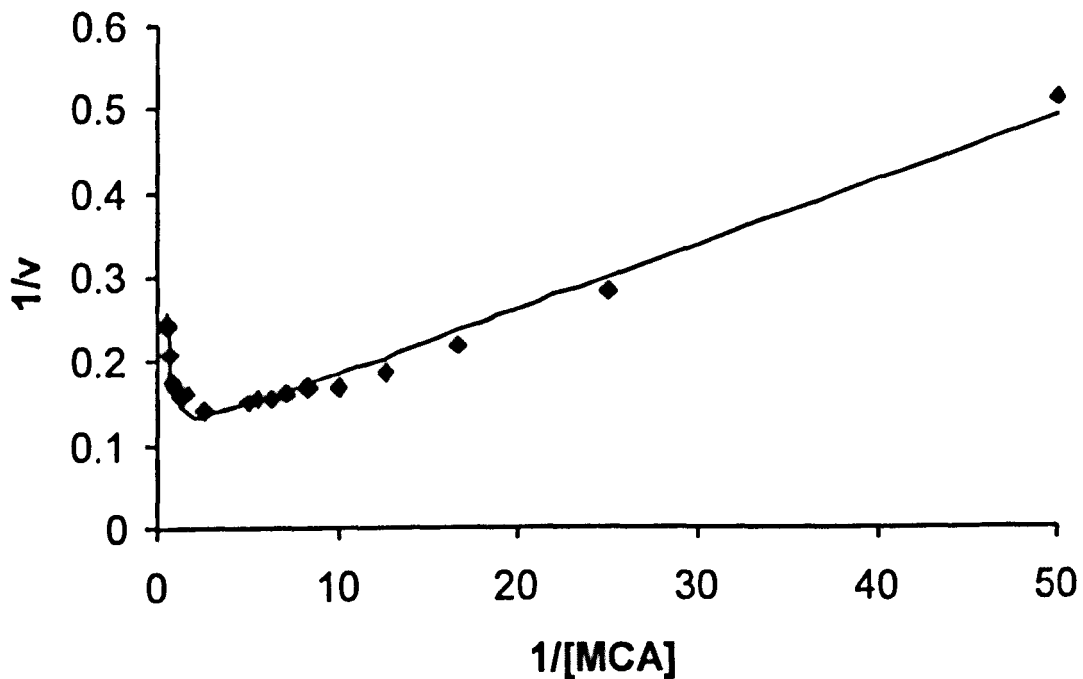


Figure 5-14: Fae-1 activity profile against MCA using a range of substrate concentrations between 0.02 - 2 mM. Graph A is plotted as substrate concentration (mM) versus activity (U ml⁻¹). Graph B is plotted as a double reciprocal plot of substrate concentration (1/mM) versus activity (1/U ml⁻¹).

5.3. OVER-EXPRESSION OF FAE-1 MUTANTS IN PICHIA PASTORIS

5.3.1. Production of Fae-1 mutants by site-directed mutagenesis

The Fae-1 sequence contains two potential motifs that fulfil the consensus G-X-S-X-G, which is highly conserved in serine esterases (Blow, 1990; Brenner, 1988). Based on the conservation of the sequence environment around these putative active sites serine residues (serine 91 and serine 118), with the *P. funiculosum* and the *A. awamori* esterase sequences, we predict the active site of the enzyme to be centred on serine 118 (G-T-S-S-G) (Figure 5-15). However, in order to determine experimentally whether serine 118 or serine 91 is a member of the catalytic triad, we decided to independently mutate Fae-1 by replacement of serine residue 91 and 118 with alanine. After replacement of the nucleophile with a neutral alanine residue it is likely that the catalytic site will lose its reactivity and the substrate will not be processed.

5.3.1.1. Site-direct mutagenesis

The site-directed mutations were performed by inverse-PCR on plasmid DNA (pCR[®]2.1-TOPO[®] /*fae-1*), using mutated primers specific to *fae-1* (Table 2-5) (Chapter 2, section 2.5.2.3.). The ligated PCR fragment was transformed into *E. coli* and plasmid DNA was sequenced. The cDNAs of *N. crassa* S91A and S118A Fae-1 mutants, containing the Fae-1 native signal sequence, were then isolated from the pCR[®]2.1-TOPO[®] cloning vector by restriction endonuclease digestion with *Bam*III-*Not*I and sub-cloned into the *Bam*III-*Not*I digested pPIC3.5K expression vector. The resulting expression vectors (pPIC3.5K/S91A/*fae-1* and pPIC3.5K/S118A/*fae-1*) were linearised as described before with *Dra*I restriction enzyme, to allow gene replacement at *AOX1* locus, and used to transform *Pichia pastoris* using the electroporation method.

					1	12	22
PfFAEB	MAIPLVLVLAWLLPVVLA-----				ASLTQVNNFGDN	PGSLQMYIYV	
NcFae-1	MLPRTLGLALTAATGLC-----				ASLQQVTNWGSN	PTNIRMITYV	
ACEA	MLLSTHLLFVITTLVTSLLHPIDGHAVKRS				GSLQQVTDGFDN	PTNVGMYIYV	
	32	42	52	62	72		
PfFAEB	PNKLASKPAI	IVAMHPCGGS	ATEYYGMYDY	HSPADQYGYI	LIYPSATRDY		
NcFae-1	PDKLATKPAI	IVALHGCGGT	APSWYSGTRL	PSYADQYGFY	LIYPGTENMS		
ACEA	PNNLASNPGI	VVAIHICTGT	GPGYYGDSPI	GTLSEQYGFY	VIYPPSPYSG		
	82	92	102	112	122		
PfFAEB	NCFDAYSSAS	LTHNGGSDSL	SIVNMVKYVI	STYGADSSKV	YMTGSSSGAI		
NcFae-1	NCWGVNDPAS	LTHGAGGDSL	GIVAMVNYTI	AKYNADASRV	YVMGTSSSGM		
ACEA	GCWDVSSQAT	LTHNGGGNSN	SIANMVTWTI	SKYGADSSKV	FVTGSSSGAM		
	132	142	152	162	172		
PfFAEB	MTNVLGAYYP	DVFAAGSAFS	GMPYACLYGA	GAADPIMSNQ	TCSQGQIQHT		
NcFae-1	MTNVMAATYP	EVFEAGAAYS	GVAHACFAGA	ASATPFSPNQ	TCARGL-QHT		
ACEA	MTNVMAATYP	ELFAAATVYS	GVSAGCFYSN	TNQVVG LNS-	TCAQGDVITT		
	182	192	202	212	222		
PfFAEB	GQQWAAAYVHN	GYPGYTGQYP	RLQMWHGTA	NVISYADLGQ	EISQWTTIMG		
NcFae-1	PEEWGNFVRN	SYPGYTGRRP	RMQIYHGLA	NLVYPRCAME	ALKQWSNVLG		
ACEA	PEHWASIAEA	MYSGYSGSRP	RMQIYHGSII	TTLYPQNYEE	TCKQWAGVFG		
	232	242	252	262	272		
PfFAEB	LSFTGNQTNT	PLSGYTKMVY	GDGSKFQAYS	AA-GVG FVP	TDVSVVLDWFG		
NcFae-1	VEFSRNVSGV	PSQAYTQIVY	GDGSKLVGYM	GA-GVG VAP	TNEQVMLKFFG		
ACEA	YDYSAPEKTE	ANTPQTNYET	TIWGDSLQGI	FATGVG TVP	IHGDKDMEWFG		
	282	292	302	312	322		
PfFAEB	ITSGTTTTTTT	PTTTPTTSTS	PSSTGGCTAA	HWAQCGGIGY	SGCTACASPYT		
NcFae-1	LIN*						
ACEA	FA*						
	332						
PfFAEB	CQKANDYYSQ	CL*					

Figure 5-15: Peptide sequence identity between esterases from *P. funiculosum* (PfFAEB) (Kroon *et al.*, 2000), *A. awamori* (ACEA) (Koseki *et al.*, 1997), and *N. crassa* (NcFae-1) (Crepin *et al.*, *in press(a)*). The signal sequence is in italic and the catalytic triad is shown in orange, with the putative nucleophilic serine in red within the consensus sequence G-X-S-X-G in blue. Grey boxes show identical amino acids. The extended *P. funiculosum* sequence features a C-terminal cellulose-binding domain preceded by a linker region (underlined). An asterisk represents the stop codon.

5.3.2. Expression, purification and characterisation of the recombinants Fae-1 mutants

His⁺ transformants resulting from the *Pichia* transformations were screened for replacement of the *AOX1* gene. 22 out of 260 transformants tested for pPIC3.5K/S91Afae-1 and 20 out of 312 for pPIC3.5K/S118Afae-1 were of the Mut^S phenotype. All the His⁺ Mut^S transformants were screened for recombinant protein expression in small-scale cultures along with *P. pastoris* clone 10 expressing the wild-type recombinant Fae-1 as a positive control for expression. Culture supernatants were analysed by SDS-PAGE for secreted protein products. Fifteen pPIC3.5K/S91Afae-1 and nine pPIC3.5K/S118Afae-1 transformants produced a major secreted protein band of approximately 35 kDa (Figure 5-16). N-terminal protein sequencing was performed in order to check the identity of the recombinant proteins, which were confirmed to be Fae-1.

One clone for each transformation was retained for large-scale expression in order to purify and characterise the recombinant enzymes. The recombinant Fae-1 mutant proteins were purified from the culture supernatants using a butyl sepharose fast flow 4 hydrophobic interaction chromatographic column as before, and the recombinant enzymes eluted between 0.65 - 0.45 M ammonium sulphate. Table 5-5 presents the enzyme yields after purification.

Table 5-5: Purification of *N. crassa* Fae-1 mutants secreted by *P. pastoris*.

Recombinant enzyme	Activity prior purification ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Activity after purification ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Yield (%)	Protein after purification ($\mu\text{g ml}^{-1}$)	Total protein for 100 % recovery (mg L ⁻¹)
S91A-Fae-1	2.059 (33 ml)	0.197 (242 ml)	70	32	335
S118A-Fae-1	Nd*	Nd	-	17	-

*Nd: non detectable

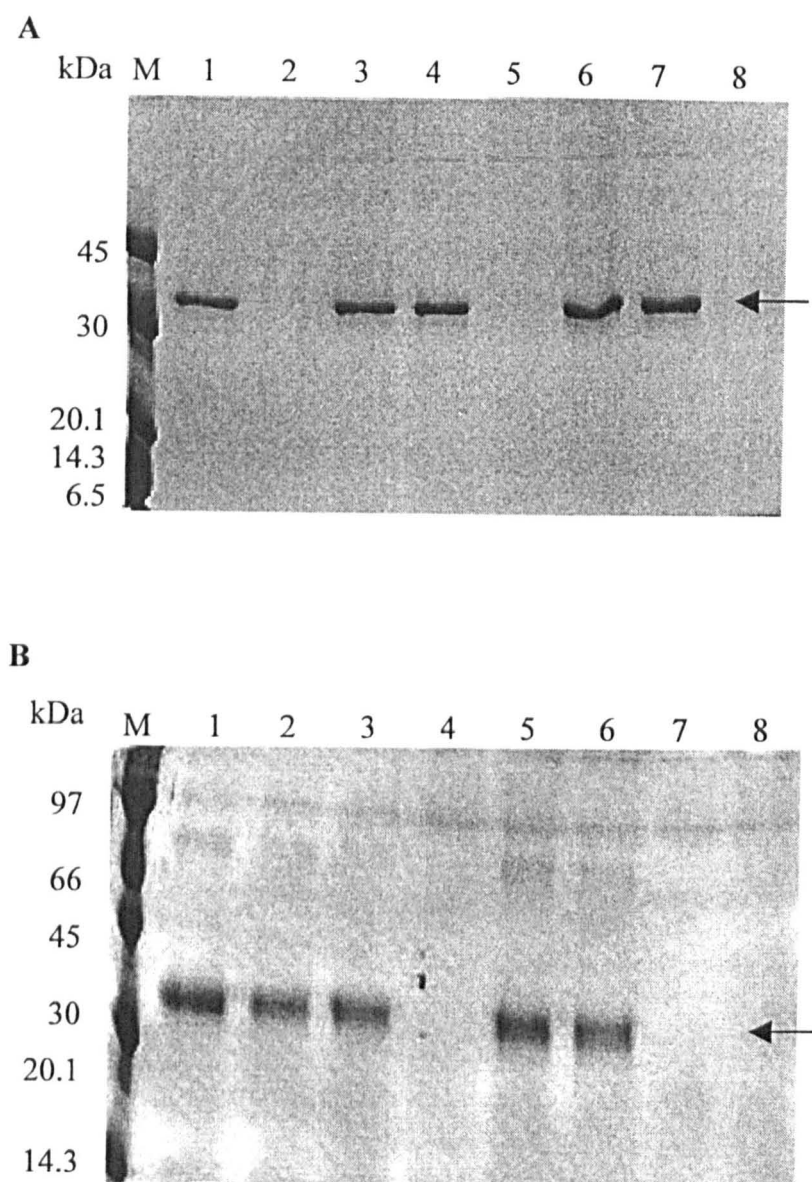


Figure 5-16: SDS-PAGE to analyse the expression of Fae-1 mutants in *P. pastoris* small-scale cultures. M: standard protein molecular weight in kDa. Lane 1: wild type Fae-1 positive control of expression. Lanes 2-8: culture supernatants of pPIC3.5K/S91Afae-1 transformants (A) and pPIC3.5K/S118Afae-1 transformants (B).

Pichia supernatants were assayed for activity against the four synthetic methyl esters substrates MCA, MFA and MSA, using spectrophotometric and HPLC methods. Feruloyl esterase activity was detected for S91A mutant but no trace of activity could be detected for the protein S118A (Table 5-5). The S91A mutant, however, seems to be altered in its kinetic capacity. The K_m was similar but the K_i was higher and the V_{max} lower than for the recombinant wild type Fae-1 (Figure 5-17 and Table 5-6).

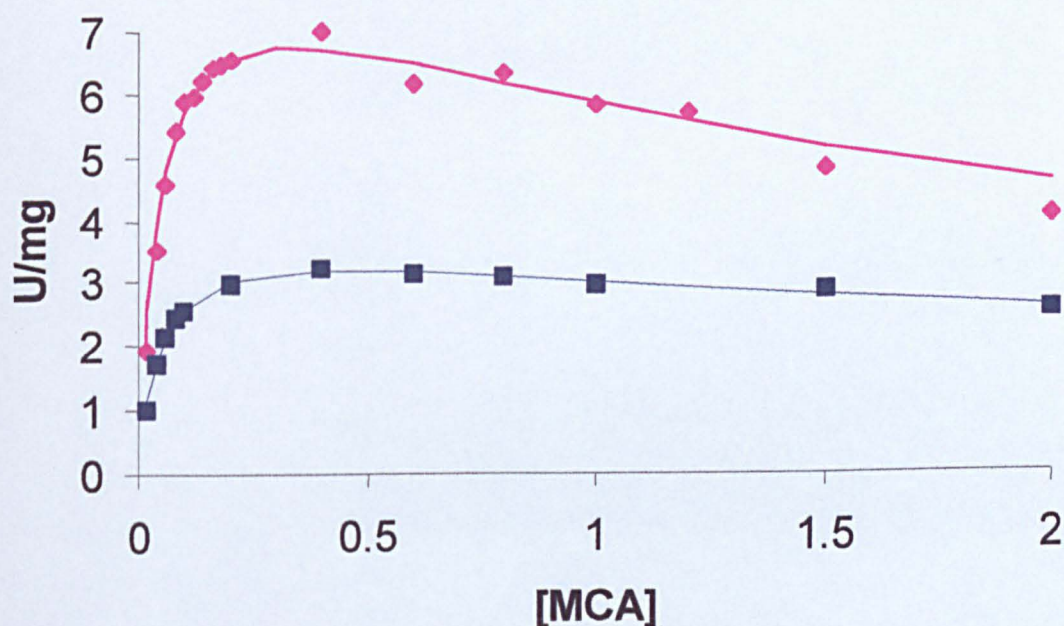


Figure 5-17: Comparison between the activity profile of wild type (pink curve) and S91A mutant (blue curve) Fae-1 against MCA, using a range of substrate concentrations between 0.02 - 2 mM. Graph is plotted as substrate concentration versus activity (U mg^{-1}).

Table 5-6: Catalytic constants of S91A Fae-1 mutant compared to wild type.

Substrate	Fae-1					S91A-Fae-1				
	V_{\max}^{\dagger} (U mg ⁻¹)	K_i (mM)	K_m (mM)	k_{cat}/K_m^{\ddagger}	χ^2	V_{\max} (U mg ⁻¹)	K_i (mM)	K_m (mM)	k_{cat}/K_m	χ^2
MFA	8.9	2.8	0.25	21	0.067	0.7	4.2	0.23	2	0.038
MpCA	20.8	2	0.021	580	0.082	2.5	8.5	0.02	74	0.009
MCA	8.2	2.5	0.048	100	0.003	4	4	0.05	46	0.036
MSA	Nd*	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd

*Nd: activity non detected. [†]1 U is defined as the amount of enzyme (mg) required to release 1 μ mol of free acid per minute. [‡]Expressed in mM⁻¹ s⁻¹.

5.4. DISCUSSION

The *fae-1* cDNA, containing the native signal sequence, was cloned and expressed in *Pichia pastoris* using the expression vector pPIC3.5K. A representative transformant producing high levels of secreted recombinant protein was selected for production of recombinant Fae-1, and used in large-scale culture for Fae-1 purification and characterisation. The yield of secreted protein was 210 mg l⁻¹ in buffered complex medium and the recombinant protein was recovered as a single peak following hydrophobic interaction chromatography. A clear advantage to the use of the *P. pastoris* expression system, is the high yield of secreted recombinant protein against a background of very limited host protein secretion, which allows for rapid purification of the target protein (Juge *et al.*, 2001).

The substrate recognition of the recombinant Fae-1 was characterised, and as expected of a Type-B feruloyl esterase, Fae-1 shows high catalytic efficiency for the hydrolysis of the methyl esters of caffeate and *p*-coumarate and no activity detectable

against methyl sinapate. The catalytic efficiency of *N. crassa* Fae-1 to hydrolyse Ara₂F was low ($12.87 \text{ mM}^{-1} \text{ sec}^{-1}$) compared to *P. funiculosum* FAEB ($530 \text{ mM}^{-1} \text{ sec}^{-1}$) (Kroon *et al.*, 2000) but was 112-fold greater than that of *A. niger* cinnamic acid esterase (CinnAE) (Kroon *et al.*, 1997b) and *P. equi* cinnamoyl ester hydrolase (CEH or EstA') (Fillingham *et al.*, 1999). The low turnover observed for Ara₂F may reflect the need for longer feruloylated oligosaccharides to ensure optimal catalytic activity. The acetyl esterase activity of Fae-1 was investigated. Even though Fae-1 was defined as feruloyl esterase because of its ability to efficiently hydrolyse hydroxycinnamic esters, the enzyme was also able to hydrolyse acetylated substrates but at a significantly lower efficiency. The ability of Fae-1 to hydrolyse α -naphthyl acetate appears to be the highest activity measured for a feruloyl esterase on this substrate ($3 \text{ s}^{-1} \text{ mM}^{-1}$), although the catalytic efficiency is 30 to one thousand-fold less than recorded for true acetyl xylan esterases (Basaran and Hang, 2000; Blum *et al.*, 1999; Degraffi *et al.*, 2000; Linden *et al.*, 1994; McDermid *et al.*, 1990a). The activity of Fae-1 against chlorogenated substrate was investigated using the 5-O-caffeoyl ester of quinic acid (chlorogenic acid). In the chlorogenated substrate, caffeic acid is ester-linked to quinic acid and the metabolism of chlorogenic acid occurs via hydrolysis to caffeic and quinic acids (Figure 5-18). The activity of Fae-1 against chlorogenic acid is 3.75-fold lower than for methyl caffeate, suggesting that the presence of quinic acid influences the substrate recognition and prevents correct alignment of the substrate ester bond in the Fae-1 active site required for optimum activity (Kroon *et al.*, 1997a). Fae-1 was also capable of releasing ferulic and *p*-coumaric acids from cell wall material. This is the first report of a feruloyl esterase able to release *p*-coumaric acid from non pre-treated cereal-derived material. In addition, Fae-1 displays high catalytic efficiency against the methyl ester of *p*-coumarate ($580 \text{ mM}^{-1} \text{ s}^{-1}$), suggesting that *p*-coumarate is indeed the best substrate for Fae-1.

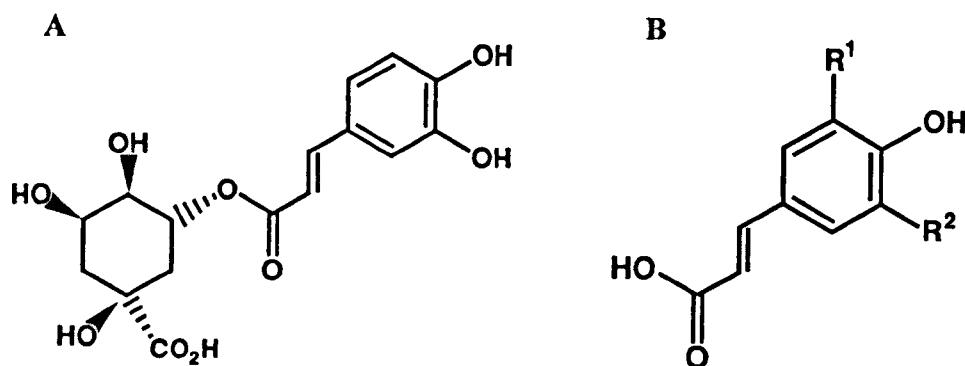


Figure 5-18: Structure of (A) 5-O-caffeoyl-quinic acid (chlorogenic acid) and (B) caffeic acid ($R^1 = \text{OH}$, $R^2 = \text{H}$).

Kinetic studies performed on the recombinant Fae-1 with the synthetic monomeric substrates MCA, MFA, and MpCA revealed substrate inhibition at higher substrate concentrations. These data were related to the equation with respect to a substrate inhibition (Equation 5-2). It is therefore possible for a second substrate molecule to bind to the enzyme-substrate complex ES (E: enzyme; S: substrate) to produce an inactive complex SES. The mechanism would be analogous to that usually considered for uncompetitive inhibition with a non-productive substrate-binding site in addition to the productive binding site. Certain fungal feruloyl esterases have been characterised as modular enzymes containing a catalytic domain linked to a non-catalytic domain such as a specific carbohydrate-binding module (CBM) (Coutinho and Henrissat, 1999). Modular esterases from *P. funiculosus* (Kroon *et al.*, 2000), *P. equi* (Fillingham *et al.*, 1999), *T. reesei* (Margolles-Clark *et al.*, 1996) were shown to contain a non-catalytic cellulose-binding domain (CBD). CBDs act to bring the catalytic site spatially closer to its substrate and to create a microenvironment where substrate is easily accessible. An enzyme subject to substrate inhibition cannot bear such a binding domain without compromising its catalytic activity, even when the substrate may be limiting in the wider environment. In the light of this information it is perhaps not surprising that the Fae-1 sequence does not feature a recognisable

CBD (Figure 5-15). In the absence of a CBD, concentration dependent substrate inhibition could be utilised by microorganisms to regulate substrate conversion. The enzyme activity would be attenuated in presence of high environmental substrate concentrations in order to prevent accumulation of toxic products that could not be immediately metabolised. Indeed, phenolic acids are used to inhibit microbial growth. We may speculate that the *N. crassa* enzyme has evolved to feature concentration dependent substrate inhibition in order to regulate the activity of a secreted esterase working remote from the organism. In contrast, concentration dependent substrate inhibition would be an ineffective way for microorganisms to regulate the activity of enzymes carrying CBDs because of the very intimacy of the contact between the enzyme and its potential substrate. The *N. crassa* genome also contains an ORF encoding a modular enzyme (chapter 4, section 4.2.2.), which may be identified as a putative feruloyl esterase with a fungal-type cellulose-binding domain (45 % identity with Fae-1 from *N. crassa*; 44 % identity with FAEB from *P. funiculosum*). *N. crassa* has therefore the genetic capacity to produce more than one Type-B feruloyl esterase and as consequence of their modular structure the organism can tailor its response to the substrate availability in the environment. In the present study substrate inhibition has been detected against synthetic substrates, which may be structurally similar to the products released by the action of other host enzymes. However, it would be of interest to know if higher order structures released from plant cell wall will produce substrate inhibition, in which case the organism may have evolved its enzymes not only to avoid toxicity but to regulate the rate at which a complex substrate is dismantled. In microbial communities, the ability of an organism to tolerate the actions of its neighbours while maintaining its own nutritional requirement, will determine the species succession in the environment. It is notable that in this respect *N. crassa* is an opportunist, being the first to colonise a nutrient source and complete its life cycle before giving way to other species.

The Fae-1 sequence features two peptide motifs (GX SXG) characteristic of the serine esterase family (Blow, 1990; Brenner, 1988; Warshel *et al.*, 1989). Of the two candidate motifs, the sequence environment of serine 118 was found to be conserved with other members of the serine esterase family, such as *P. funiculosum* FAEB and *A. awamori* ACEA (Koseki *et al.*, 1997; Kroon *et al.*, 2000). In contrast the

amino acid sequences surrounding serine 91 are not well conserved. Therefore, we predict the active site of the enzyme to be centred on serine 118 (G-T-S-S-G) (Figure 5-15). In order to test this hypothesis, both putative serine residues (serine 118 and serine 91) were independently mutated and replaced with an alanine residue. The two Fae-1 mutant proteins were expressed in *P. pastoris* and purified from culture supernatants. Enzyme activity was detected only with the S91A mutant. The S118A mutant was totally inactive suggesting that serine 118 is indeed one of the key amino acids involved in the catalytic reaction, most likely to act as the nucleophilic residue. The activation of the nucleophilic serine occurs through a charge-relay system, which involves catalytic histidine and aspartic acid residues (Warshel *et al.*, 1989). By replacing the serine residue with an alanine, we have disturbed the charge-relay system resulting in the inability of the enzyme to metabolise the substrate and by consequence no product is released. Serine residue 118 was expected to be the nucleophilic residue of the active site based on the comparison of the *N. crassa* Fae-1 amino-acid sequence with nine other sequences of serine esterases (Kroon *et al.*, 2000), where serine, histidine, and asparagine residues, which comprise the catalytic triad, are conserved.

The S91A mutant retained activity but was altered in its kinetic capacity. As described above, Fae-1 is subject to substrate inhibition, implying that a second molecule of substrate can bind to the enzyme-substrate complex resulting in a sterile complex. This mechanism is likely to be similar to that described for uncompetitive inhibition where the inhibitor decreases the value of V_{\max} with no corresponding effect on that of K_m . In other words, the inhibitor interferes with the catalytic properties of the enzyme without affecting the substrate binding affinity. Following the mutation of the serine residue 91 to alanine, we observed that the value of K_m remained similar but K_i increased and the V_{\max} reduced as compared with the wild type Fae-1, affecting the catalytic constant of the S91A enzyme. This would suggest that serine 91 participates in the binding of the inhibitory substrate molecule and also that serine 91 may directly or indirectly influence the catalytic competence of the enzyme. Hypothetically the protein could contain two distinct substrate-binding pockets, one high affinity site providing the framework for catalytic processing (K_m) and one low affinity site (K_i) that is sterile. Serine 91 could interact with both

pockets. This interaction would be essentially to confer stability on the substrate-enzyme complex during the catalytic process, and thereby influence the catalytic rate of the reaction. Once the substrate concentration increases and reaches the K_i value, a second molecule of substrate can bind to the enzyme-substrate complex. This would destabilise the interactions existing between serine 91 and the first substrate molecule lodged in the high affinity site. Serine 91 would then preferentially interact with the inhibitory substrate molecule resulting in a decrease in the kinetic velocity. By replacing serine 91 with alanine, we have decreased the substrate affinity for the non-catalytic substrate-binding pocket, and in doing so we may have also amended interactions between serine 91 and substrate bound within the catalytic pocket, resulting in a diminution in the affinity for a second substrate molecule to bind the enzyme-substrate complex (K_i increased) and a decrease in the catalytic rate reflected by a lower V_{max} . Serine 91 is separated by 22 residues in the linear peptide chain from the presumptive nucleophile, which could place the two serine residues within relative close proximity within the three-dimensional structure. Close spatial proximity between the two binding pockets would allow serine 91 to interact with both substrate molecules. However, in order to verify this speculation and the existence of two substrate-binding pockets, the next step would be to determine the structure of *N. crassa* Fae-1.

In conclusion, we have shown that the purified product of the *fae-1* gene, encoding a non-modular feruloyl esterase, is subject to substrate inhibition. A search of the *N. crassa* genome sequence has revealed three further ORFs with protein sequences similar to Fae-1 (chapter 4, section 4.2.2.), including a putative enzyme carrying a CBD. This would suggest that feruloyl esterases have evolved to operate over a wide range of substrate availability but at the same time have an innate ability to modulate their catalytic activity in respect of the catabolic requirements and product sensitivities of the organism that secretes them. We have also demonstrated that serine 118 is essential for the catalytic ability of Fae-1. The sequence motif GTSSG contains the nucleophilic serine that is conserved amongst the feruloyl esterase class. Serine 91, however, is not essential for the catalytic reaction but seems to influence the inhibition constant value as well as the catalytic properties of the enzyme.

CHAPTER 6. EXPRESSION AND CHARACTERISATION OF RECOMBINANT TSFAEC IN *PICHTA PASTORIS*

6.1. INTRODUCTION

Feruloyl esterases constitute an interesting group of enzymes that have the potential for use over a broad range of applications in the agri-food industries. There is considerable scope to utilise feruloyl esterases to process otherwise waste agricultural materials into valuable products. As a consequence these enzymes have become a focus for research to identify and produce enzymes capable of acting on a diverse range of substrates at levels commensurate with their applications to commercial processes (Juge *et al.*, 2001).

Tal. stipitatus grown in liquid culture in the presence of sugar beet pulp resulted in the production of two extracellular feruloyl esterase activities. One of these activities, dubbed Type-C feruloyl esterase (TsFAEC), was found to be active at a similar rate against the four synthetic methyl ester substrates (MCA, MFA, MpCA, and MSA). The activity of TsFAEC was measured against several benzoic and cinnamic acid methyl esters to determine whether the enzyme exhibits a broader substrate utilisation than the known Type-A or Type-B feruloyl esterases from *A. niger* (Kroon *et al.*, 1997a). TsFAEC exhibits a specificity which compliments both the Type-A and Type-B feruloyl esterases in that it hydrolyses the methyl esters of cinnamic, 3,4-dimethoxycinnamic, isoferulate and chlorogenic acid (Kroon *et al.*, 1997a). No activity was detected against the benzoic acid methyl esters. These results suggest that TsFAEC is an example of a new type of fungal feruloyl esterase with an hitherto unseen ability to hydrolyse a broad spectrum of methyl hydroxycinnamates. We are therefore in possession of an enzyme, which may offer benefits to a broader range of current industrial applications and the potential for new commercial applications.

In order to provide a rapid and ready source of enzyme for industrial applications and to enable further biochemical characterisation of the enzyme, we decided to try and over-express TsFAEC in the *Pichia pastoris* expression system, as it has successfully been used to secrete active *Neurospora crassa* Fae-1 feruloyl esterase (Chapter 5) and AnFAEA (Juge *et al.*, 2001).

6.2. OVER-EXPRESSION OF FAEC IN PICHIA PASTORIS

The *Tal. stipitatus* feruloyl esterase gene (*faeC*, GenBank Accession Number: AJ505939) was translated and submitted to the SignalP World Wide Web server, designed to predict the presence and location of signal peptide cleavage sites in amino acid sequences (Nielsen *et al.*, 1997). The program predicts that a cleavage site (at 90.7 % confidence) occurs between amino acid 19 (A) and 20 (D) (SHA↑DDS: ↑ indicating site of cleavage) to generate the peptide sequence DDSSRENFS. However, the N-terminal amino acid sequence determined for mature FAEC purified from *Tal. stipitatus* was NFSN, some six amino acids internal to the site of the computer prediction.

6.2.1. Utilisation of different signal sequence to replace the native FAEC signal peptide

In order to investigate any functional influence that these six extra amino acids may have on the enzyme activity or the capacity of *P. pastoris* to secrete the FAEC protein, expression vectors were constructed to produce FAEC with or without these additional N-terminal six amino acids (Table 6-1). Moreover, we appended alternative signal peptide to the N-terminus of FAEC to assess their relative secretion efficiency, and if they had any influence on the selection of the signal peptide cleavage site by *P. pastoris*. The first expression vector contained *faeC* cDNA directed by its native signal sequence (pPIC3.5K/*faeC* expression vector, Table 6-1). The *N. crassa* Fae-1 secretion signal had already proven to efficiently secrete active feruloyl esterase in *P. pastoris* (Chapter 5), and was therefore utilised to produce recombinant TsFAEC with N-terminal sequences starting at DDS or NFS directed by the expression constructs pPIC3.5K/ss1-DDS*faeC* or pPIC3.5K/ss1-NFS*faeC*

respectively (Table 6-1). In addition to these constructions, we used the secretion signal of the α -factor from *Saccharomyces cerevisiae* that is routinely used to direct the secretion of heterologous proteins from *Pichia* (pPIC9/ α -NFS*faeC*, Table 6-1) (Bake *et al.*, 1984; Cregg *et al.*, 1993).

6.2.1.1. Sub-cloning into the expression vector

The *faeC* gene was amplified from genomic DNA using a proofreading DNA polymerase, sub-cloned into pCR[®]4-TOPO[®] and sequenced. cDNA amplification was then carried out by inverse-PCR performed on pCR[®]4-TOPO[®]/*faeC*-gene plasmid DNA using the TsFAEC-Int-F forward and the TsFAEC-Int-R reverse primers positioned on either side of the intron (Table 6-2) (Chapter 2, section 2.4.2.3.). The primers were designed to introduce a silent restriction site (*Nsi*I: ATGCAT) into the self-ligated inverse-PCR product, which was used as a selective marker to differentiate specific PCR products from the others and selected clones were sequenced. The cDNA fragment, containing the *Tal. stipitatus* FAEC native signal sequence, was isolated from the cloning vector pCR[®]4-TOPO[®]/*faeC* by restriction endonuclease digestion with *Sna*BI-*Not*I and ligated into the vector pPIC3.5K digested with the same restriction enzymes, resulting in the expression construct pPIC3.5K/*faeC* (Chapter 2, section 2.5.2.4.1.).

The pPIC3.5K/ss1-NFS*faeC* and pPIC3.5K/ss1-DDS*faeC* expression vectors were constructed using the signal sequence from the Fae-1 feruloyl esterase of *Neurospora crassa* (GenBank Accession Number: AJ293029), fused to TsFAEC N-terminal sequences starting with NFS or DDS. Single stranded DNA was first PCR amplified from pCR[®]4-TOPO[®]/*faeC* plasmid DNA using either TsFAEC-ss1-NFS or TsFAEC-ss1-DDS to add the *N. crassa* Fae-1 secretion signal sequence before commencing the *faeC* reading frame at the codons NFS or DDS (Table 6-2). Double strand DNA was then amplified using the primers ss1-F and C16R (Table 6-2). The constructions were sequenced before sub-cloning into the *Sna*BI-*Not*I sites of the expression vector pPIC3.5K (Table 6-1) (Chapter 2, section 2.5.2.4.2.).

The pPIC9/ α -NFS*faeC* expression vector was constructed using the *S. cerevisiae* α -factor signal sequence, which is present in the pPIC9 vector sequence. The cDNA was amplified by PCR of pCR[®]4-TOPO[®]/*faeC* DNA using TsFAEC- α -NFS/TsFAEC-C16R primers. The TsFAEC- α -NFS primer was designed in frame with the 3'-end of the α -factor signal sequence, to fuse the TsFAEC reading frame starting with the codons for NFS (Table 6-2). The construction was then sequenced, and sub-cloned in frame with the α -factor signal sequence within the *XhoI*-*NotI* digested pPIC9 expression vector (Table 6-1) (Chapter 2, section 2.5.2.4.3.).

The resulting expression vectors were linearised with *DraI* restriction enzyme, allowing gene replacement at *AOX1* locus, and used to transform *Pichia pastoris* using the electroporation method.

Table 6-1: Alternative signal peptides used in the expression of FAEC by *P. pastoris*

Vector construction	Amino acid sequences of secretion signal sequence fused to mature FAEC peptide sequence	N-terminal amino acid sequence of the secreted protein*
pPIC3.5K/ <i>faeC</i>	MLTSAILLLTLGVQLSHA^a↑ DDS SRE ^b ↑ NFSNRCDQLAKEIHIPNVTV NFVE.....	DDSSRENFSN.....
pPIC3.5K/ss1-NFS <i>faeC</i>	MLPRTLGLALTAATGLC^a↑ NF SNRCDQLAKEIHIPNVTVN.....	NFSNRCDQLA.....
pPIC3.5K/ss1-DDS <i>faeC</i>	MLPRTLGLALTAATGLC^a↑ DD SSRENFSNRCDQLAKEIHIP.....	DDSSRENFSN.....
pPIC9/ α -NFS <i>faeC</i>	MRFPSIFTAVLFAASSALAAPVNTTT EDETAQIPAEAVIGYSDLEGDFDVA VLPFSTNNGLLFINTTIAAIAAKE EGVSLEKR^{a,c}↑ EA ^d EA ^d NFS NRCDQLAKEIHIPN.....	EAEANFSNRC.....

*The first 10 amino acids of the recombinant proteins N-terminus were determined by protein sequence analysis using a Sequencer Model 473A. ↑ Indicates a site of cleavage determined experimentally. ^a Indicates the position of the cleavage site recognised by *P. pastoris* secretion machinery. ^b Indicates the position of the native FAEC N-terminal peptide sequence. ^c Indicates the Kex2 protease cleavage site. ^d Indicates the Ste13 protease cleavage site. Signal sequences are in bold.

Table 6-2: Names and sequences of oligonucleotides used in PCR reactions

Primer names	Primer nucleotide sequence ^a
TsFAEC-Int-F	5'- <u>GC ATC</u> CAG TAT GTA GAC ATG GC-3'
TsFAEC-Int-R	5'- <u>A TCC</u> TGC GAG ACC ACC ATT AC-3'
TsFAEC-ss1-NFS	5'-AAA GCT CGA <u>GTA CGT ATG</u> TTG CCC AGA ACA TTG CTC GGG CTC GCC CTC ACC GCG GCC ACA GGC CTC TGT AAC TTC TCA AAT CGA TGC GA-3'
TsFAEC-ss1-DDS	5'-AAA GCT CGA <u>GTA CGT ATG</u> TTG CCC AGA ACA TTG CTC GGG CTC GCC CTC ACC GCG GCC ACA GGC CTC TGT GAT GAT TCG TCC CGC GAG AAC-3'
TsFAEC-ss1-F	5'-AAA GCT CGA <u>GTA CGT ATG</u> TTG C-3'
TsFAEC-C16R	5'-AAA <u>AGC GGC CGC</u> TAC TAC AAA ATA CAT TTC CAC C-3'
TsFAEC-α-NFS	5'-AA <u>GCT CGA GAA</u> AAG AGA GGC TGA AGC TAA CTT CTC AAA TCG ATG CGA CC-3'
TsFAEC-S166A-F	5'- <u>C GCA</u> ACA GGC GGA CGA CAA GGG-3'
TsFAEC-S166A-R	5'- <u>CA</u> GCC GAG ATA GTA TGA TTT G-3'
TsFAEC-S465A-F	5'- <u>CTA GGT</u> AGT GGG CCG GGA GC-3'
TsFAEC-S465A-R	5'- <u>GGC</u> AGC ACC ACG AAT ATA CTC CG-3'

^aThe restriction sites introduced in the primer sequences are underlined (*Sna*BI: TA CGTA; *Not*I: GC GGC CGC; *Xho*I: CTCGAG; *Nsi*I: ATGCAT; *Avi*II: TGCGCA; *Bln*I: CCTAGG). Altered codons are in bold. Chapter 2, section 2.5.2.4.

6.2.2. Expression of the recombinant FAEC in *Pichia pastoris*

Transformants were selected by their ability to grow on histidine-deficient medium, and His⁺ transformants were assessed for their Methanol Utilisation phenotype (Mut phenotype). Mut⁺ transformants were screened for expression in small-scale cultures under methanol induction, along with one colony transformed with the parental vector as a control of the background protein secretion levels. Culture supernatants of the *P. pastoris* transformants were analysed by SDS-PAGE for protein expression to reveal that each of the four constructions generated a major secreted protein of approximately 72 kDa (Figure 6-1 A). The theoretical molecular weight for the mature FAEC protein is 55 340 Da and the molecular weight of the native enzyme has been estimated by SDS-PAGE at 66 kDa. The additional mass would suggest that the FAEC product, like other exogenous proteins expressed by *P. pastoris*, is glycosylated, which is in agreement with the six potential N-linked glycosylation sites predicted from the FAEC sequence (Chapter 3, Figure 3-7). The 72 kDa product was absent in the vector control. N-terminal sequencing was performed in order to check the nature of the secreted protein and to identify the cleavage site recognised by the *P. pastoris* secretion machinery. The native FAEC secretion signal was cleaved as predicted by the SignalP program to produce the N-terminal sequence DDS (directed from pPIC3.5K/*faeC*). The Fae-1 signal peptide was cleaved as observed in *N. crassa* and predicted by SignalP to generate recombinant FAEC enzymes with N-terminal sequences of either DDS or NFS directed from the vectors pPIC3.5K/ss1-DDS*faeC* or pPIC3.5K/ss1-NFS*faeC*, respectively (Table 6-1). However, when using the α -factor secretion signal (pPIC9/ α -NFS*faeC* vector), four amino acids (EAEA) remained in front of the predicted signal peptidase cleavage site due to inefficient cleavage at the preceding Ste13 protease site (Table 6-1). To confirm these proteins were enzymatically active, culture supernatants were assayed for activity and the recombinant enzymes were found to be active against the four synthetic substrates (MCA, MFA, MSA and MpCA). The substrate utilisation of the recombinant FAEC was identical to that observed for the native FAEC protein (Chapter 3, Table 3-1).

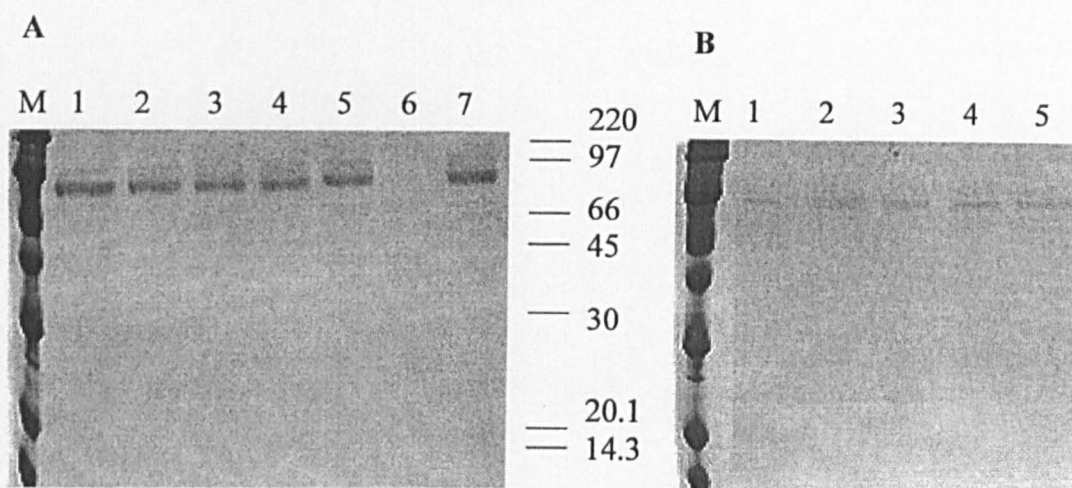


Figure 6-1: Secretion and purification of the recombinant FAEC proteins. A) SDS-PAGE analysis of supernatants from small-scale cultures of *P. pastoris* transformants expressing recombinant FAEC stained with Coomassie brilliant blue. M: molecular weight marker (kDa). Lanes 1-5 and 7: recombinant FAEC secreted using pPIC9/ α -NFS*faeC*, pPIC3.5K/ss1-NFS*faeC*, pPIC3.5K/ss1-DDS*faeC*, pPIC3.5K/S166A-*faeC*, pPIC3.5K/S465A-*faeC*, and pPIC3.5K/*faeC*, respectively. Lane 6: negative control. B) SDS-PAGE following purification of recombinant FAEC proteins. M: standard molecular weight marker (kDa). Lanes 1-5: purified recombinant α -NFS-FAEC, ss1-NFS-FAEC, S166A-FAEC, S465A-FAEC, and FAEC, respectively.

6.2.3. Purification and characterisation of the recombinant FAEC

FAEC proteins representing the three alternative N-terminal sequences generated were purified from culture supernatants. Transformants carrying either pPIC9/ α -NFS*faeC*, or pPIC3.5K/ss1-NFS*faeC*, or pPIC3.5K/*faeC* were cultured in 50 ml BMMY and the cell supernatants fractionated by butyl sepharose fast flow 4 hydrophobic interaction chromatography. In each case a single peak of activity

against MCA (0.1 mM) was eluted between 0.6 - 0.4 M ammonium sulphate. The active fractions were pooled and the purity of the samples assessed by SDS-PAGE (Figure 6-1 B). All three versions of the FAEC protein produced single migrating bands. Table 6-3 presents the enzyme yields of these products after purification. Protein concentrations of the recombinant FAEC enzymes were calculated from the absorbance at 280 nm using the FAEC extinction coefficient determined from the amino acid sequence ($90\,960\text{ M}^{-1}\text{ cm}^{-1}$) and show that the nature of the secretion signal has had little effect on the secretion yield (Table 6-3).

Table 6-4 presents the kinetic constants (k_{cat} , K_{m} , $k_{\text{cat}}/K_{\text{m}}$) calculated from initial rates of the recombinant FAEC against hydroxycinnamic acid esters, using the theoretical molecular weight for the mature FAEC protein (55 340 Da). Substrate concentrations between 0.02 mM and 1.0 mM, were employed to estimate the kinetic constants from the Michaelis-Menten equation (Equation 5-1). The K_{m} for methyl caffeate could not be determined in these experiments as the rate of reaction with this substrate remained maximal even at the lowest substrate concentration (0.005 mM) for which the product was measurable using the HPLC method. What can be concluded is that the K_{m} for this substrate is low, $< 0.005\text{ mM}$. The extra amino acids at the front of the FAEC sequence did not influence the performance of the enzyme, since the kinetic constants remained similar for all the recombinant enzymes regardless of the N-terminal peptide sequence.

Table 6-3: Purification of recombinant *Tal. stipitatus* FAEC secreted by *P. pastoris*

Recombinant enzyme	Activity prior purification ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Activity after purification ($\mu\text{mol min}^{-1} \text{ml}^{-1}$)	Yield (%)	Protein after purification ($\mu\text{g ml}^{-1}$)	Total protein for 100 % recovery (mg L^{-1})
FAEC	2.31 (15 ml)	0.17 (197 ml)	89	15.7	232
ss1-NFS-FAEC	2.53 (37 ml)	0.20 (252 ml)	55	20.8	260
α -NFS-FAEC	3.26 (35 ml)	0.28 (242 ml)	59	18.3	214
S166A-FAEC	N.D.*	N.D.	-	12.5	-
S465A-FAEC	3.86 (33 ml)	0.28 (265 ml)	58	21.5	297

* N.D. non detectable

Table 6-4: Enzyme properties of recombinant *Tal. stipitatus* FAEC

Substrate	k_{cat} (s^{-1})	k_m (mM)	k_{cat}/k_m ($\text{mM}^{-1} \text{s}^{-1}$)	χ^2
MFA	9.65	0.04	323	0.013
MpCA	9.55	0.01	746	0.002
MSA	9.26	0.37	25	0.520
MCA	-	< 0.005	> 1065	-

6.3. OVER-EXPRESSION OF FAEC MUTANTS IN *PICHA PASTORIS*

The predicted sequence of FAEC contains two potential peptide motifs that fulfil the consensus sequence G-X-S-X-G that features the characteristic serine residue that is generally conserved in the catalytic mechanism of the esterase family (Brenner, 1988; Dodson and Wlodawer, 1998). These serine residues (serine 166 present in the motif GCSTG and serine 465 present in GASLG. Chapter 3, Figure 3-7) are candidates for the nucleophilic residue forming the catalytic triad of FAEC (numbered from the mature N-terminal asparagine determined for native FAEC). The putative serines have been mutated at each of these sites and replaced by alanine. The corresponding enzymes were over-expressed in *P. pastoris* to identify the candidate nucleophilic residue responsible for catalysing the enzymatic reaction.

6.3.1. Production of FAEC mutants by site-directed mutagenesis

In order to ascertain which of these serines may act as the nucleophilic residue, they were independently mutated to replace the serine residue with neutral alanine. Site-directed mutagenesis was performed by inverse-PCR on plasmid DNA (pCR[®]4-TOPO[®]/*faeC*) and self-ligation of the inverse-PCR product (Chapter 2, section 2.5.2.5.). Primers (TsFAEC-S166A-F/TsFAEC-S166A-R and TsFAEC-S465A-F/TsFAEC-S465A-R, Table 6-2) were positioned on either side of the mutation point: serine 166 or serine 465, respectively (with the first amino acid being the asparagine identified from the N-terminal sequence of native FAEC). They were designed to replace the serine residue with alanine and to introduce a silent restriction site (*Avi*II and *Bln*I, respectively, Table 6-2) into the self-ligated inverse-PCR product, which was used as a selective marker. The constructions were sequenced before sub-cloning in the *Sna*BI-*Not*I digested expression vector resulting in pPIC3.5K/*faeC*-S166A and pPIC3.5K/*faeC*-S465A vectors.

6.3.2. Expression, purification and Characterisation of the mutants

The cDNAs of *Tal. stipitatus* S166A and S465A FAEC mutants, containing the FAEC native signal sequence, were sub-cloned into the pPIC3.5K expression vector and expressed in *P. pastoris* under the control of the inducible alcohol oxidase promoter. The recombinant FAEC proteins were purified from culture supernatants using a butyl sepharose fast flow 4 hydrophobic interaction chromatographic column (Figure 6-1 B). Ferulic acid esterase activity was detected for S465A but no trace of activity could be detected for protein S166A (Table 6-3) using both spectrophotometric and reverse-HPLC methods. The kinetic behaviour of the active enzyme S465A proved to be identical to that of the wild type FAEC (Figure 6-2).

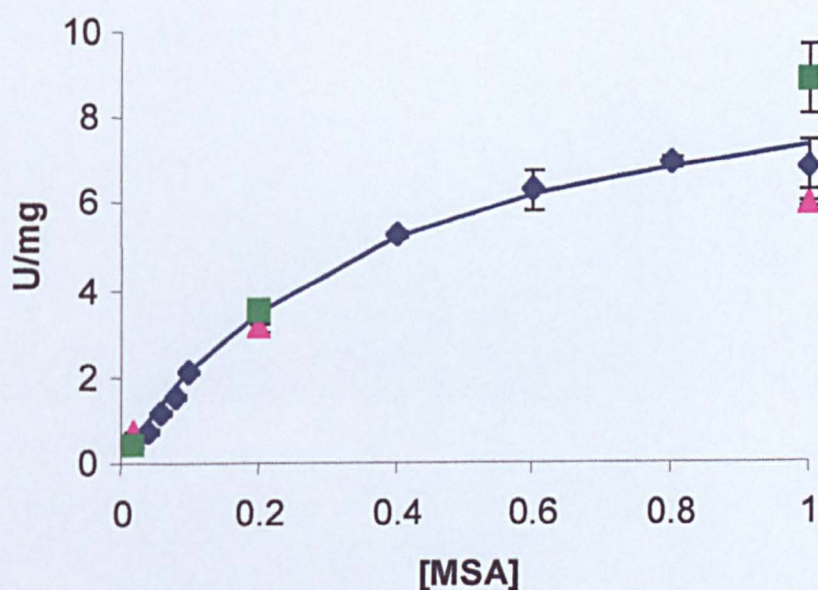


Figure 6-2: Comparison between the activity profile of recombinant FAEC proteins: DDS-FAEC in blue, NFS-FAEC in pink and S465A-FAEC mutant in green. The activity was measured against MSA, using a range of substrate concentrations between 0.02 – 1.0 mM. Graph is plotted as substrate concentration versus activity (U/mg).

6.4. DISCUSSION

Secretion of *Tal. stipitatus* FAEC by *P. pastoris* directed by its native signal sequence leads to the production of a recombinant protein with six extra amino acids in front of the sequence as compared to the N-terminal peptide sequence determined for the FAEC secreted by *Tal. stipitatus*. The position of the cleavage is consistent with that predicted by the SignalP program at high confidence. If the signal peptidase of *Tal. stipitatus* behaves in a similar manner, then it seems likely that the native enzyme may have undergone secondary cleavage during the growth period prior to harvesting. We have investigated the secretion by *P. pastoris* of recombinant FAEC proteins produced with and without these six extra amino acids by making use of the *N. crassa* Fae-1 signal peptide. The additional amino acids present at the N-terminus of the sequence do not influence the catalytic capacity of the enzyme. We conclude that N-terminal trimming of the secreted product is unlikely to affect the function of the enzyme. FAEC is harvested from *Tal. stipitatus* culture supernatant after 7 days fermentation with SBP. Under these conditions the enzyme is likely to be subject to proteolysis by alternative enzymes secreted by the host. The N-terminal residues under these conditions appear dispensable. The nature of the signal sequence did not appreciably alter the secretion yield with the *Tal. stipitatus* FAEC signal sequence reaching 232 and 297 mg L⁻¹, *N. crassa* Fae-1 secreted 260 mg L⁻¹ and using *S. cerevisiae* α -factor secretion signal produced 214 mg L⁻¹.

The FAEC sequence features two peptide motifs that match the consensus sequence G-X-S-X-G, which has been shown to contain the serine responsible for nucleophilic attack in the conserved mechanism of serine esterases (Blow, 1990; Brenner, 1988; Dodson and Wlodawer, 1998; Warshel *et al.*, 1989). Of the two candidate motifs, the sequence environment of serine 166 was found to be conserved with other members of the serine esterase family, such as *A. oryzae* tannase and *A. niger* FAEB (Figure 6-3) (de Vries *et al.*, 2002b; Hatamoto *et al.*, 1996). In contrast the amino acid sequences surrounding serine 465 are not well conserved. It is therefore perhaps more likely that the serine 166 region contains the functional nucleophile. In order to test this hypothesis, both putative serines (serine 166 and serine 465) were

independently mutated and replaced with an alanine residue. As the nucleophilic serine is replaced by a neutral residue, the catalytic site will lose its ability to hydrolyse the substrate, which should not be processed. The two FAEC mutant proteins were expressed in *P. pastoris* and purified from culture supernatants. Feruloyl esterase activities were measured using spectrophotometric and HPLC methods. Enzyme activity was detected only with the S465A mutant. The S166A mutant was below the HPLC detection limit suggesting that serine 166 is indeed the nucleophilic residue responsible for catalysing the enzymatic reaction. Serine 166 is an important component of the catalytic structure generally identified as the Ser-His-Asp triad present in serine esterase families, that together participate in a charge-relay reaction (Dodson and Wlodawer, 1998; Prates *et al.*, 2001). By replacing serine 166 with alanine, it is likely that we have amended the triad's polarisation, disturbed the charge-relay system to prevent the formation and subsequent breakdown of a covalent acyl-enzyme intermediate, in a similar fashion to that observed for several other superfamily members (Brady *et al.*, 1990; Brenner, 1988; Dodson and Wlodawer, 1998; Jaeger *et al.*, 1999; Ollis *et al.*, 1992; Prates *et al.*, 2001). It is therefore anticipated that the replacement of serine by alanine will result in an inert enzyme. In contrast mutant S465A retained the enzyme activity and did not appear affected by the amino acid modification. Serine 465 is clearly not a critical component of the catalytic reaction.

<i>Tal. stipitatus</i> FAEC feruloyl esterase (AJ505939)	(183)	-KSY ¹⁶⁶ Y ¹⁶⁷ LG ¹⁶⁸ CS ¹⁶⁹ TG ¹⁷⁰ GR ¹⁷¹ RG ¹⁷² FK-	(331)
<i>A. niger</i> FAEB feruloyl esterase (AJ309807)	(177)	-YSY ¹⁶⁶ Y ¹⁶⁷ LG ¹⁶⁸ CS ¹⁶⁹ TG ¹⁷⁰ GR ¹⁷¹ QGY ¹⁷² Q-	(328)
<i>X. campestris</i> tannase precursor (AE012315)	(176)	-YSYFFGASNGGRDALI-	(384)
<i>Agrobacterium tumefaciens</i> tannase (AE008948)	(199)	-RVYFQGGSTGGREGLT-	(338)
<i>Acinetobacter</i> sp. chlorogenate esterase (L05770)	(107)	-RSYAGGTSNGGRHAMM-	(458)
<i>A. oryzae</i> tannase (D63338)	(187)	-YTY ¹⁶⁶ YEG ¹⁶⁷ CS ¹⁶⁸ DGGREG ¹⁶⁹ MS-	(385)

Figure 6-3: Alignment of the sequences around the essential serine residue 166 of *Tal. stipitatus* FAEC with other members of the serine esterase family. The sequences are aligned with respect to the putative catalytic serine residues shown in bold. The numbers in parentheses indicate the numbers of residues N-terminal and C-terminal to the alignment in each protein.

We have shown that the enzymatic activity of the recombinant FAEC feruloyl esterase from *Tal. stipitatus*, is insensitive to the addition of extra amino acids to the N-terminus of the protein. *Pichia pastoris* can support the secretion of FAEC using alternative secretion peptides without unduly altering the efficiency. This may be facilitated by the tolerance of FAEC to changes in its N-terminal sequence. We have formally demonstrated that serine 166 is essential for the catalytic ability of FAEC. The sequence motif GCSTG contains the nucleophilic serine that is conserved amongst the ferulic acid esterase class. The use of the *P. pastoris* expression system to produce large quantities of recombinant wild type and mutant FAEC enzymes that may be purified efficiently will enable the initiation of structure-function studies and provide a basis for commercial exploitation.

CHAPTER 7. CLASSIFICATION OF FERULOYL ESTERASES: IDENTIFICATION OF A TYPE-D ENZYME FROM *N. CRASSA*

7.1. PUTATIVE FERULOYL ESTERASES CLASSIFICATION

Feruloyl esterases have previously been classified as Type-A (e.g. *A. niger* FAEA) and Type-B (e.g. *A. niger* CinnAE and FAEB, *P. funiculosum* FAEB) based on their specificity for the aromatic moiety, specificity for the linkage to the primary sugar, and ability to release ferulic acid dehydrodimers from esterified substrates (de Vries *et al.*, 2002b; Kroon *et al.*, 1999; Kroon *et al.*, 2000). At present, the lack of highly conserved sequences within the sequenced esterases has not permitted further classification for the feruloyl esterases other than that their primary amino acid sequences place them in family 1 of the carbohydrate esterase classification (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/>). Carbohydrate esterases catalyse the de-O or de-N-acetylation of substituted saccharides. Since an ester is an acid plus an alcohol, two classes are considered: those in which the sugar plays the role of the "acid", such as pectin methyl esters and those in which the sugar behaves as the alcohol, such as in acetylated xylan. A number of possible reaction mechanisms may be involved: the most common is a Ser-His-Asp catalytic triad catalysed deacetylation which is analogous to the action of lipases and serine proteases but other mechanisms such as a Zn^{2+} catalysed deacetylation could also be considered for some group members (Coutinho and Henrissat, 1999).

Using literature reports of the characteristics of the feruloyl esterases, together with the growth substrate requirements for crude cell wall materials leading to the expression of these enzymes by various microbes, provided a basis upon which to organise the feruloyl esterases into different classes. From these comparisons the following feruloyl esterases: *P. funiculosum* FAEB (Accession number AJ291496); *N. crassa* Fae-1 (AJ293029); *P. fluorescens* XYLD (X58956); *P. funiculosum* FAEA

(AJ312296); *P. equi* EstA (AF164516); *A. niger* FAEA (AF361950); *Tal. stipitatus* FAEC (AJ505939); *A. niger* FAEB (AJ309807) could be divided into four putative sub-classes termed Type-A, B, C and D (Table 7-1). Members of each sub-classes show similar activity profiles against four synthetic substrates (MFA, MCA, MSA, MpCA) and similar abilities to release diferulate or not. Moreover, these classes are likely to reflect the substrate availability of natural plant cell walls as they are preferentially induced by their respective organisms when grown in the presence of similar substrates. Finally it can be demonstrated that members of the same activity class also share a high degree of amino acid sequence identity.

In order to investigate further the feruloyl esterase sub-classification, a phylogenetic tree was drawn on the basis of primary sequence identity between selected plant cell wall acting esterases and other sequence related enzymes such as lipases and xylanases. The phylogenetic tree of the cladogram type was constructed by the neighbour-joining method (Saitou and Nei, 1987), using the CLUSTAL W program available at www.ebi.ac.uk/clustalw/ (Thompson *et al.*, 1994). A cladogram is a branching tree assumed to be an estimate of a phylogeny where the branches are of equal length, which shows common ancestry but does not indicate the amount of evolutionary time separating taxa. The phylogenic tree indicates that feruloyl esterases have diverged in three main branches, within which they have evolved along discrete lines to form sub-classes of specialised enzymes (Figure 7-1).

Table 7-1: Classification of feruloyl esterases based on primary amino acid sequence identity, specificity for hydroxycinnamic acid methyl esters, ability to release diferulic acids from model and complex substrates and inducible plant cell wall materials.

Type-A	Type-B	Type-C	Type-D
<i>A. niger</i> FAEA	<i>P. funiculosum</i> FAEB <i>N. crassa</i> Fae-1	<i>A. niger</i> FAEB <i>Tal. stipitatus</i> FAEC	<i>P. funiculosum</i> FAEA <i>Pir. equi</i> EstA <i>Ps. fluorescens</i> XYLD
<u>Preferentially induced on</u> WB	<u>Preferentially induced on</u> SBP	<u>Preferentially induced on</u> SBP – WB	<u>Preferentially induced on</u> WB
<u>Hydrolysis of methyl esters of</u> MFA MSA MpCA	<u>Hydrolysis of methyl esters of</u> MFA MCA MpCA	<u>Hydrolysis of methyl esters of</u> MFA MSA MpCA MCA	<u>Hydrolysis of methyl esters of</u> MFA MSA MpCA MCA
<u>Release of diferulic acid</u> Yes (5-5')	<u>Release of diferulic acid</u> No	<u>Release of diferulic acid</u> No	<u>Release of diferulic acid</u> Yes (5-5')
<u>Sequence similarity with</u> Lipase	<u>Sequence similarity with</u> CE family 1 Acetyl xylan esterase	<u>Sequence similarity with</u> Chlorogenate esterase Tannase	<u>Sequence similarity with</u> Xylanase

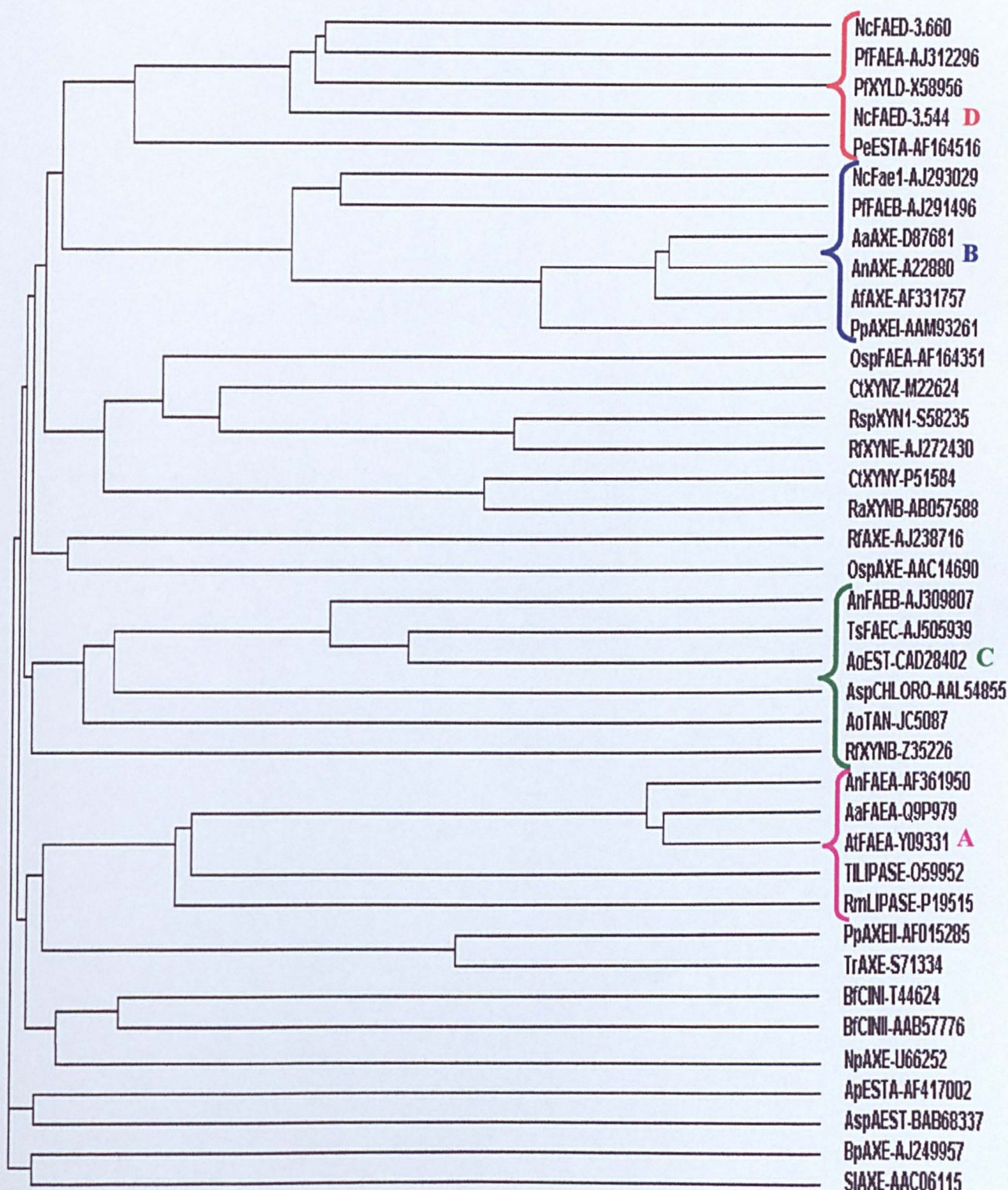
This phylogenetic tree also shows that TsFAEC and AnFAEB are closely related and have evolved from the same root. As a consequence we suggest that these enzymes belong to the same sub-class of feruloyl esterases, termed Type-C, despite differences in their activity profile such as the reported substrate specificity of

AnFAEB on methyl hydroxycinnamates, which initially suggested this enzyme to be a Type-B feruloyl esterase (de Vries *et al.*, 2002b). Not surprisingly, the acetyl xylan esterase II from *Penicillium purpurogenum* (Accession number AF015285) and the catalytic domain of AXE I from *Trichoderma reesei* (S71334), which share 67 % sequence identity (Gutierrez *et al.*, 1998), seem to have evolved via a similar pathway from a common ancestor. The need to conserve structural features of these enzymes will influence the degree of amino acid conservation and reveal the evolutionary relationships between them. On the other hand, enzymes such as XynY and XynZ from *C. thermocellum* (Accession numbers P51584 and M22624, respectively), FaeA from *Orpinomyces* sp. PC-2 (AF164351) and Xyn1 from *Ruminococcus* sp. (S58235), which have previously been shown to share sequence identity (Blum *et al.*, 2000), are closely related. In parallel, the phylogenetic tree shows that although the feruloyl esterases have some common roots, in some cases they may be more sequence related to a variety of other enzymes such as lipases, acetyl xylan esterases or xylanases than they are to each other.

Although the four sub-classes termed Type-A, B, C, and D have been ascribed on the basis of their synthetic substrate preferences, the common ability of plant cell wall substrates to preferentially provoke the expression of these feruloyl esterases and supported by primary sequence comparisons, the phylogenetic analysis also suggests that these are probably yet further unnamed enzyme sub-classes than can only be discriminated on the basis of their primary amino acid sequence identity. No further correlation could be established due to the lack of comparable enzyme activity data. Nevertheless, in an attempt to classify the feruloyl esterases with the available data, the feruloyl esterases belonging to carbohydrate esterase family 1 may be subdivided into four classes termed Type-A, B, C, and D as shown in Figure 7-1. Future acquisition of protein and predicted protein sequences and complementary enzyme activity data will enable the substantiation of other enzymes classes recognised in the phylogenetic analysis. These data will in turn provide a better understanding of the complex evolutionary relationship between the feruloyl esterases.

Figure 7-1: Cladogram of enzymes of the feruloyl esterase family and relatives. Sequence names with their Accession Numbers are shown on the right of the tree. NcFAED-3.660, *Neurospora crassa* FAED-3.660 feruloyl esterase; PfFAEA, *Penicillium funiculosum* FAEA (Furniss *et al.*, 2001); PfXYLD, *Pseudomonas fluorescens* subsp. *Cellulosa* XYLD or esterase D (Ferreira *et al.*, 1993); NcFAED-3.544, *Neurospora crassa* FAED-3.544 feruloyl esterase; PeESTA, *Piromyces equi* ESTA (Fillingham *et al.*, 1999); NcFae-1, *Neurospora crassa* Fae-1 (Crepin *et al.*, *in press(a)*); PfFAEB, *Penicillium funiculosum* FAEB (Kroon *et al.*, 2000); AaAXE, *Aspergillus awamori* AXE (Koseki *et al.*, 1997); AnAXE, *Aspergillus niger* AXE (de Graaff *et al.*, 1992); AfAXE, *Aspergillus ficuum* AXE (Chung *et al.*, 2002); PpAXEI, *Penicillium purpurogenum* AXE I (Gordillo and Eyzaguirre, 2002); OspFAEA, *Orpinomyces* sp. PC-2 FAEA (Blum *et al.*, 2000); CtXYNZ, *Clostridium thermocellum* XYNZ (Blum *et al.*, 2000); RspXYN1, *Ruminococcus* sp. Xyn1 (Arakaki *et al.*, 1997); RfXYNE, *Ruminococcus flavefaciens* XYNE (Aurilia *et al.*, 2000); CtXYNY, *Clostridium thermocellum* XYNY (Blum *et al.*, 2000); RaXYNB, *Ruminococcus albus* XYNB (Nagamine *et al.*, 2001); RfAXE, *Ruminococcus flavefaciens* AXE (Aurilia *et al.*, 2000); OspAXE, *Orpinomyces* sp. PC-2 AXE A (Blum *et al.*, 1999); AnFAEB, *Aspergillus niger* FAEB (de Vries *et al.*, 2002b; Kroon *et al.*, 1996); TsFAEC, *Talaromyces stipitatus* FAEC (Crepin *et al.*, *in press(b)*); AoEST, *Aspergillus oryzae* selective esterase (Stergaard *et al.*, 2002); AspCHLORO, *Acinetobacter* sp. Chlorogenate esterase (Hartnett *et al.*, 1990); AoTAN, *Aspergillus oryzae* tannase (Hatamoto *et al.*, 1996); RfXYNB, *Ruminococcus flavefaciens* XYNB (Zhang *et al.*, 1994); AnFAEA, *Aspergillus niger* FAEA (Faulds and Williamson, 1994); AaFAEA, *Aspergillus awamori* FAEA (Koseki *et al.*, 1998; Koseki *et al.*, 1999); AtFAEA, *Aspergillus turbingensis* FAEA (de Vries *et al.*, 1997); TILIPASE, *Thermomyces lanuginosus* lipase (Boel *et al.*, 1998); RmLIPASE, *Rhizomucor miehei* lipase (Boel *et al.*, 1988; Derewenda *et al.*, 1992); PpAXE, *Penicillium purporogenum* acetylxylan esterase II (Gutierrez *et al.*, 1998); TrAXE, *Trichoderma reesei* acetylxylan esterase I (Margolles-Clark *et al.*, 1996); BfCINI, *Butyrivibrio fibrisolvens* E14 cinnamoyl ester hydrolase I (Dalrymple *et al.*, 1996); BfCINII, *Butyrivibrio fibrisolvens* E14 cinnamoyl ester hydrolase II

(Dalrymple and Swadling, 1997); NpAXE, *Neocallimastix patriciarum* AXE (Dalrymple *et al.*, 1997); ApESTA, *Aspergillus parasiticus* ESTA esterase (Yu, 2001); AspAEST, *Acinetobacter* sp. AEST esterase (Suzuki *et al.*, 2001); BpAXE, *Bacillus pumilus* AXE (Degrassi *et al.*, 2000); SIAXE, *Streptomyces lividans* AXE (Shareck *et al.*, 1995).



7.2. IDENTIFICATION AND EXPRESSION OF A TYPE-D FERULOYL ESTERASE FROM *N. CRASSA*

The presence of *N. crassa* genomic sequences predicting protein products with significant amino acid identity to reported feruloyl esterases is consistent with the idea that *N. crassa* is capable of producing more than one type of feruloyl esterase, and consistent with the feruloyl esterase activity measurements and time-course mRNA expression analysis presented in Chapter 4 of this thesis. These sequence identities reflect the diversity and the multiplicity of esterase activities produced by *N. crassa* when grown on plant cell wall materials, and offer a number of genes to investigate in terms of the substrate specificities of the enzymes so encoded.

7.2.1. Identification of putative feruloyl esterase genes

In order to identify genes encoding putative feruloyl esterases, sequences of feruloyl esterases available from the general databank were compared to the translated sequences present in the *N. crassa* genomic databank (<http://www-genome.wi.mit.edu/>). The sequences identified in the *N. crassa* genome were then conversely compared to the sequences available in the general databank (NCBI) using the BLAST program at NCBI (Altschul *et al.*, 1997; Zhang and Madden, 1997). The translation of two sequences (contig 3.544 and 3.660) from *N. crassa* genome database show primary sequence identity with FAEA feruloyl esterase from *Penicillium funiculosum* (43 %) and XYLD from *Pseudomonas fluorescens* (41 %), (Figure 7-2). These sequence relationships identified two *N. crassa* genomic sequences as candidate feruloyl esterase genes, these were provisionally named *faeD*-3.544 and *faeD*-3.660.

7.2.2. Sequence analysis

The open reading frames of the *faeD*-3.544 and *faeD*-3.660 genes encode proteins of 291 and 303 amino acids respectively. The computer program SignalP (Nielsen *et al.*, 1997), predicts the respective *FAED*-3.544 and *FAED*-3.660 proteins to contain

secretory signal peptide of 25 and 17 amino acids. Based on these predictions the calculated masses of the mature proteins are 28 598 Da for FAED-3.544 and 29 685 Da for FAED-3.660. Analysis of the protein sequences reveals the presence of two motifs in FAED-3.544 and three in FAED-3.660 that are characteristic of the serine esterase family (G-X-S-X-G) (Brenner, 1988; Dodson and Wlodawer, 1998). In each case only one of these motifs (G-F-S-Y-G) is centred on a serine within a conserved region also present in *P. funiculosum* FAEA and *P. fluorescens* XYLD sequences, as well as other members of the serine esterases family (Fillingham *et al.*, 1999). Based on the conservation of the sequence environment around these putative active sites serine residues it may be predicted that the active site of the enzyme FAED-3.544 is centred on serine 132 and that of FAED-3.566 is centred on serine 141 (G-F-S-Y-G, block 2 in Figure 7-2). The translated sequences, however, do not feature a recognizable cellulose-binding domain, unlike those observed for the *P. funiculosum* and *P. fluorescens* esterases (Ferreira *et al.*, 1993).

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3.660  MYLFGSLLLG LACLTEA--- -----
3.544  MAGLHSRLTT FLLLLLSALP AIAAA-----
XYLD
PfFAEA MVKSYIIGAF VLELASVVLG QQSLWGQCGG TGWTGPTTCV SGACCQEQNP

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3.660  -----LS TTIPSPHARA TSRSPPCGGSA
3.544  -----APSSGCGKG
XYLD                                     (324) GLGKP
PfFAEA YYSQCIQGNC SPASSTSSTS STKTTTTTSAS STTTSTSASG TSLSSGCGKA

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3.660  PTITSGVKSL T-VNGKQRQF TIRVPQNYQK DTTYKVIITGL HWVGGTMDQV
3.544  PTLRNGQTVT TNINGKSRRY TVRLPDNYNQ NNPYRLIFLW HPLGSSMQKI
XYLD   RTNLQNGT-V IQSGGTQRSY ILDAPGNYNN NKPYRLVIGY HWRGGTAQDV
PfFAEA LSLKSG-TYT TTVAGQQRQY TLTLP SNYNP NKAYQLIFGY HWLGGMTGMNV

```

Block 1

```

3.660  AGGG--TTGE PWAYYGMQKM -ANESAILVA PQGLNNGWAN AGGEDIQFID
3.544  IQGEDPNRGG VLPYYGLPPL DTSKSAIYVV PDGLNAGWAN QNGEDVSFFD
XYLD   HTGQTVQRDA -WAHYGMKIL -AGDSTIFVA PQGIGNGWGN GGADVTFTD
PfFAEA VSGS----- --YGIQPL -AGDNAIFVA PQGLNNGWGN TNGDDIIFTD

```

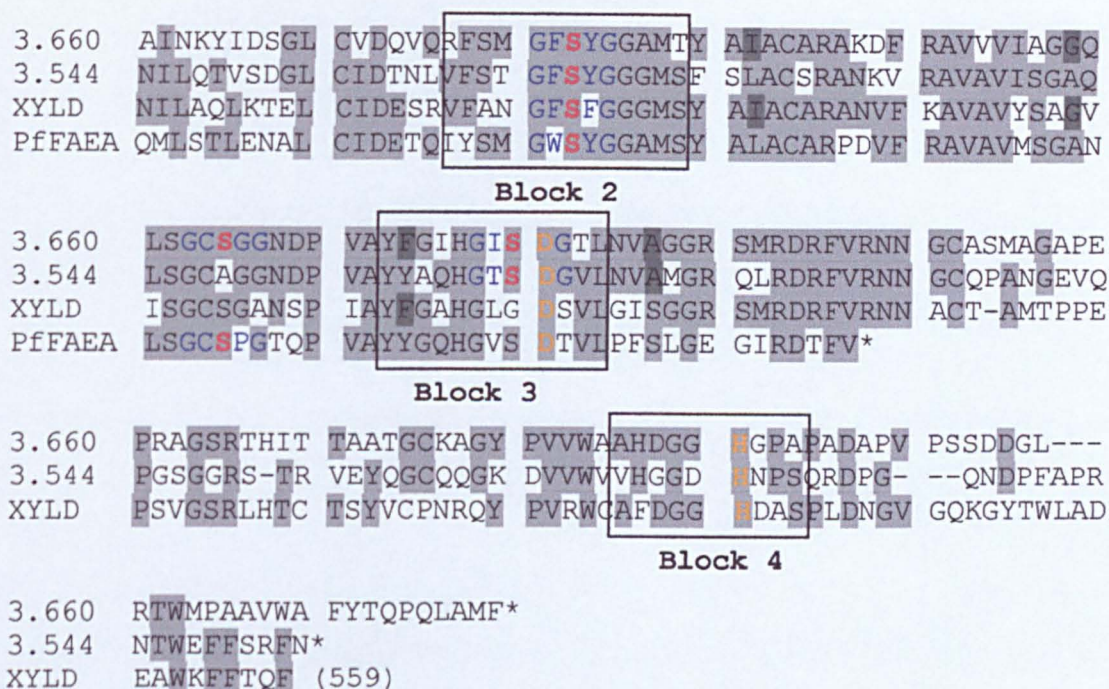



Figure 7-2: The two putative Type-D feruloyl esterases (FAED-3.544 and FAED-3.660) from *N. crassa* are aligned with the sequences of XYLD from *P. Fluorescens* subsp. *Cellulosa* (X58956, Ferreira *et al.*, 1993) and FAEA from *P. funiculosum* (AJ312296). Signal sequences are shown in italic. The PfFAEA cellulose-binding domain is underlined and the linker region is in bold script. Four blocks show conserved sequences in esterases, which contain residues that are possible components of the catalytic triad (in orange) or the oxyanion hole (Fillingham *et al.*, 1999; Jaeger *et al.*, 1999). Putative nucleophilic serines are in red within the serine esterase consensus sequence (G-X-S-X-G) in blue. The numbers in parentheses at the ends of the XYLD sequence denote the positions of the residues with respect to the full-length sequence. Asterisks mark the positions of termination codons.

Based on sequence identity, FAED-3.544 and FAED-3.660 enzymes have been identified as belonging to a new sub-class of esterases that we have designated as Type-D (Figure 7-1). As we had already Type-A, B, and C feruloyl esterase

representatives cloned and expressed in *P. pastoris* (Crepin *et al.*, 2002; Crepin *et al.*, *in press(a)*; Crepin *et al.*, *in press(b)*; Juge *et al.*, 2001), we decided to amplify the putative gene sequences from *N. crassa* DNA and attempt to express these reading frames in *Pichia pastoris*. It was considered that these additional proteins could provide evidence for yet further diversity in the roles and substrate recognition of the feruloyl esterase family.

7.2.3. Cloning and expression of Type-D feruloyl esterases in *P. pastoris*

The nucleotide sequences of *faeD*-3.544 and *faeD*-3.660 were analysed for the presence of intronic DNA with reference to the sequence conservation of the amino acid sequences and conservation of the splicing signal common to filamentous fungal introns (Bruchez *et al.*, 1993; Radford and Parish, 1997). As no introns were identified and the open reading frames of the translated sequences were not interrupted, the *faeD*-3.544 and *faeD*-3.660 ORFs were PCR-amplified from *N. crassa* genomic DNA using specific primers designed to introduce *Sna*BI and *Not*I restriction sites at 5' and 3' termini of both putative Type-D feruloyl esterase sequences (Chapter 2, Table 2-4). These fragments were cloned into the pCR®2.1-TOPO® vector and the clones DNA sequenced to ensure the absence of nucleotide changes from the sequences present in the database.

The effective *faeD*-3.544 and *faeD*-3.660 cDNA fragments were isolated from the cloning vector by restriction digest using *Sna*BI-*Not*I endonucleases and ligated into *Sna*BI-*Not*I digested pPIC3.5K expression vector under the control of the alcohol oxidase 1 promoter (*AOX1*) (Chapter 2, section 2.5.2.2.). The resulting expression vectors pPIC3.5K/*faeD*-3.544 and pPIC3.5K/*faeD*-3.660 were linearised with *Dra*I restriction enzyme, to allow gene replacement at the *AOX1* locus, and used to transform *Pichia pastoris* GS115 (*his4*) using the electroporation method. *P. pastoris* transformants were selected by their ability to grow on histidine-deficient medium. The transformation generated several hundred His⁺ transformants, which were assessed for their Methanol Utilisation phenotype (Mut phenotype).

From each transformation, eight colonies were picked and screened for protein expression and secretion under methanol induction in small-scale cultures. Culture supernatants were analysed by SDS-PAGE for protein expression. Eight *faeD*-3.544 *Pichia* transformants produced a major secreted protein product of approximately 32 kDa (Figure 7-3), whereas none of the *faeD*-3.660 *Pichia* transformants tested produced detectable recombinant protein.

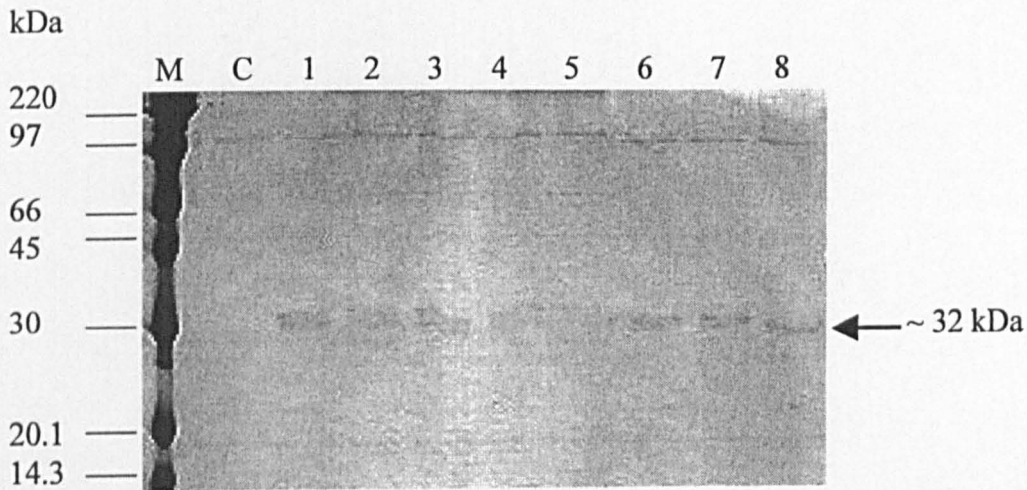


Figure 7-3: SDS-PAGE to analyse the expression of FAED-3.544 in *P. pastoris* small-scale cultures. M: standard protein molecular weight in kDa; C: vector control; lanes 1-8: His⁺ Mut^S pPIC3.5K/*faeD*-3.544 transformant clones 1 to 8.

7.2.4. Feruloyl esterase activity of the recombinant FAED-3.544

To confirm these cultures were feruloyl esterase-producing transformants, culture supernatants were assayed for activity against MCA, MFA, MpCA and MSA, using reverse-phase HPLC method (Table 7-2, assays performed at the IFR by Dr. C. Faulds) (Faulds and Williamson, 1994). The recombinant protein was found to be active against all four synthetic substrates. In addition, the ability of FAED-3.544

to release phenolic compounds from agro-industrial waste materials was tested. The enzyme was found to be able to release ferulic, *p*-coumaric and diferulic acids from wheat bran and Brewer’s spent grain in combination with xylanase from *T. viride* (Table 7-3, assays performed at the IFR by Dr. C.B. Faulds). These results confirm that the *faeD*-3.544 gene does indeed encode a feruloyl esterase.

Table 7-2: Feruloyl esterase activity of the recombinant *N. crassa* FAED-3.544 against 1 mM hydroxycinnamic acid methyl esters. Activity is expressed as mU ml⁻¹ of culture supernatant.

MFA	MSA	MCA	MpCA
77.4	83.6	44.6	45.6

Table 7-3: Release of phenolic residues from agro-industrial waste materials by *N. crassa* FAED-3.544, using 1 mU esterase with 2 U *Trichoderma viride* xylanase (Megazyme) at 37 °C for 3 hours. Values are expressed as % of the total alkali-extractible phenolics.

Plant cell wall material	Ferulic acid released	<i>p</i> -Coumaric acid released	5-5’ diferulic acid released
Wheat bran	36	23	12
Brewer’s spent grain	18	0.5	10

7.3. DISCUSSION

A new attempt to classify feruloyl esterase has been proposed based on sequence identities and similarities in esterase activity profiles against synthetic methyl esters. From this classification, four sub-classes have been characterised and termed Type-A, B, C, and D. As part of this project, a Type-B and C feruloyl esterases have already been cloned, over-expressed and characterised. In order to enlarge our panel of esterases, we undertook to clone and over-express putative Type-D feruloyl esterases from *N. crassa*.

Recombinant *N. crassa* FAED-3.544 shows activity against all four synthetic substrates as reported for the genes encoding *P. fluorescens* Type-D esterase (PfXYLD) and *Tal. stipitatus* FAEC (Crepin *et al.*, *in press(b)*; Ferreira *et al.*, 1993). Moreover, in the presence of 2 U *T. viride* xylanase, FAED-3.544 is capable of releasing ferulic and 5-5' diferulic acid from untreated wheat bran and brewer's spent grain. The ferulate and diferulate levels released are similar to those released by AnFAEA and PfXYLD, under the same conditions (Table 7-4). Broad specificity against synthetic substrate suggests that FAED-3.544 could be responsible for the broad range esterase activity detected in the later stages (days 7 - 9) of the *N. crassa* fermentation on either wheat bran or sugar beet pulp (Chapter 4). This hypothesis, which has to be verified with substantial data such as time-course mRNA expression, would support the idea that induction of an enzyme with broad substrate activity could replace the specialised enzymes later in the fermentation to complete the digestion of complex plant materials.

Table 7-4: Release of phenolic residues (FA: ferulic acid, *p*CA: *p*-coumaric acid, DiFA: 5-5' diferulic acid) from agro-industrial waste materials using 1 mU NcFAED-3.544 esterase with 2 U *Trichoderma viride* xylanase (Megazyme) at 37 °C for 3 hours, 13 mU AnFAEA or PfXYLD esterase with 2 U *T. viride* xylanase at 37 °C for 7 hours (Bartolome *et al.*, 1997a; Bartolome *et al.*, 1997b; Faulds and Williamson, 1995c). Values are expressed as % of the total alkali-extractible phenolics. ND: not determined. *C.B. Faulds, personal communication.

	AnFAEA			PfXYLD			NcFAED-3.544		
	FA	<i>p</i> CA*	diFA	FA	<i>p</i> CA	diFA	FA	<i>p</i> CA	diFA
Wheat bran	95	< 2	20	95	ND	20	36	23	12
Spent grain	27	< 2	16	27	ND	16	18	0.5	10

FAED-3.544 also appears to release *p*-coumaric acid from wheat bran and brewer's spent grain. Release of *p*-coumaric acid from cell wall materials has been previously reported for *p*-coumaroyl esterases from *Aspergillus awamori*, *Penicillium pinophilum*, and *Neocallimastix* strain MC-2 (Borneman *et al.*, 1991; Castanares *et al.*, 1992; Castanares and Wood, 1992; McCrae *et al.*, 1994). However, these measurements have been carried out on phenolic acid-substituted xylan polysaccharide isolated from wheat straw and rarely on crude cell wall materials with the exception of coastal Bermuda grass utilised to study the *p*-coumaric acid release by *Neocallimastix* strain MC-2 *p*-coumaroyl esterase (Borneman *et al.*, 1991). Therefore, this is the first report of an esterase able to release significant quantities of *p*-coumaric acid from crude cell wall materials such as wheat bran and brewer's spent grain.

Esterified phenolics present in plant cell wall materials may hinder degradation by microorganisms. The use of feruloyl esterases in pre-treated animal feed, such as

wheat straw, to remove esterified phenolics increases digestibility and relieves toxicity to ruminal bacteria (Williamson *et al.*, 1998b). As wheat straw is rich in *p*-coumaric acid esterified to lignin (Scalbert *et al.*, 1985), an enzyme able to release these *p*-coumaric residues could be attractive for the feed industry. Therefore, the ability of FAED-3.544 to release *p*-coumaric acid from plant cell wall materials in addition to ferulate and diferulate acid should be investigated using various plant cell wall materials in order to identify further potential industrial applications.

The ferulate and diferulate levels released from plant cell wall materials by FAED-3.544 are similar to those released by AnFAEA under the same conditions. However, the broad substrate specificity against methyl hydroxycinnamates as well as the ability to release 5-5' diFA and significant levels of *p*CA acid from untreated plant cell wall materials demonstrate that FAED-3.544 is different from a Type-A feruloyl esterase, which is not active against MSA, and is also different from a Type-C feruloyl esterase, which does not release dimers. Based on sequence identity FAED-3.544 has been classified as a Type-D feruloyl esterase and its activity profile, which is similar to that of PfXYLD esterase, confirmed that it indeed belongs to the Type-D feruloyl esterase class.

To conclude, this chapter results the identification, cloning and over-expression of a Type-D feruloyl esterase, which is different from previous Type-A, B, and C feruloyl esterases. The activity profile of FAED-3.544 offers new potential industrial applications and increases the diversity of feruloyl esterases available to dismantle plant cell wall materials.

CHAPTER 8. APPLICATIONS AND OUTCOMES

The preceding chapters of this thesis contain experimental results and general discussion of the data. However, in this final chapter, I will examine the industrial applications that the feruloyl esterases characterised in this project can offer, and specifically the role of these feruloyl esterases in bread making. The potential for commercial use of these feruloyl esterases and more particularly the novel *Talaromyces stipitatus* Type-C feruloyl esterase has been exploited by Biocatalysts Limited.

8.1. INTRODUCTION

Wheat endosperm, commonly referred to as flour, contains wheat pentosans, which are mainly composed of arabinoxylans that consist of a main chain of 4-linked β -D-xylopyranosyl residues, some of which carry α -L-arabinofuranosyl substituents with a few of the arabinose sugars being esterified with ferulic acid (Rouau, 1993). These arabinoxylans can be classified into two types. Water extractable or water-soluble arabinoxylan (WSAX, approximately one-third of the arabinoxylans), which form highly viscous solutions and can undergo oxidative gelation, and water-insoluble arabinoxylan (WIAX), which possess a strong water holding capacity and can be subdivided into alkali/enzyme solubilised arabinoxylans and residual unextractable arabinoxylans (Courtin and Delcour, 2001). WIAX contains more phenolic acids than WSAX, with ferulic acids mostly confined to the high molecular weight fractions of WSAX, which also contain the lowest degrees of arabinose substitution (Izydorczyk and Biliaderis, 1993). Ferulic acids are involved in the oxidative cross-linking reaction of individual arabinoxylan to form a larger network (Izydorczyk *et al.*, 1990), and cross-linking between ferulic acid and dough components such as polysaccharides and proteins is thought to be important in bread making (Hoseney and Faubion, 1981). As a consequence, the presence of feruloyl esterases in additives can either be beneficial by reducing unnecessary cross-linking or detrimental if inappropriate links are broken during dough formation.

8.2. PRELIMINARY EXPERIMENTS

These experiments have been carried out in collaboration with colleagues at IFR Norwich (Faulds *et al.*, *in press*).

In order to examine how feruloyl esterases may affect the arabinoxylan of flour, feruloyl esterases (NcFae-1, AnFAEA, and TsFAEC) belonging to three of the four sub-classes identified have been examined for their specificity and ability to act on soluble and insoluble arabinoxylans extracted from wheat flour. The enzymes have been tested either alone or in the presence of xylanases belonging to the glycosyl hydrolase family 10, which preferentially cleave in decorated regions or family 11, which preferentially cleave in unsubstituted regions (Biely *et al.*, 1997). The three feruloyl esterases used in this study are clearly more active against WSAX than WIAX with the Type-C feruloyl esterase from *Talaromyces stipitatus* performing the best, releasing 100 % of the ferulic acid in combination with a family 11 xylanase from *Trichoderma longibrachiatum*. Type-A esterase from *Aspergillus niger*, however, was the most effective in releasing ferulic acid from insoluble material using a combination of AnFAEA and the family 11 xylanases from either *T. viride* or *T. longibrachiatum*. No diferulate release was detected from WSAX with any of the feruloyl esterases and the presence of xylanase was essential for the release of 5-5' dimers from WIAX by AnFAEA only. Fae-1 Type-B feruloyl esterase from *Neurospora crassa* was the least effective performer of the three esterases tested.

Low levels of diferulic acid present in WIAX may be of considerable significance in the gel formation of dough by improving the water-holding capacity of the arabinoxylans (Rouau *et al.*, 1994). The enzyme-catalysed weight loss in the WIAX fraction causes a proportional decrease of the amount of water retained. In the presence of enzyme, degradation of WIAX is mainly due to the solubilisation of arabinoxylans, which increases substantially the arabinoxylan content of the water-extractable fraction of dough and this leads to wet and sticky doughs (Rouau *et al.*, 1994). By consequence, the selection of the correct enzymes and their utilisation at an appropriate level of addition is crucial to obtain a positive effect on the baking process. Feruloyl esterases, for example, must be generally active but preferentially

unable to release diferulates, such as observed for Type-B or Type-C feruloyl esterases. Ferulic acid is known to be involved in the interaction between arabinoxylan and gluten but also in the ability of pentosans to aggregate. Pentosan aggregation is correlated to their ferulic acid content and is highly related to their ability to reduce the extensibility of dough. Addition of free ferulic acid reduces the viscosity of WSAX due to free ferulic acid reacting with feruloylated arabinoxylan, preventing pentosan aggregation, and resulting in an increase in dough extensibility (Wang *et al.*, 2002). Moreover, release of ferulic acid by feruloyl esterases in dough will prevent unwanted oxidative cross-link formation between arabinoxylans or arabinoxylan and gluten proteins in the presence of peroxidase/H₂O₂, which prevent polymer aggregation (Oudgenoeg *et al.*, 2001).

8.3. BAKING TRIALS

Based on the results obtained for the release of ferulic acid from WSAX and WIAX by a Type-A, B and C feruloyl esterases, *Talaromyces stipitatus* Type-C feruloyl esterases, which was the most efficient at releasing ferulic acid from WSAX and did not release dimers, was selected as the most promising enzyme for use in bread making.

Preliminary baking trials have been carried out by Biocatalysts to establish the effect of different types of feruloyl esterases on bread making (Type-A, B and C). The most significant effect was observed with *Talaromyces stipitatus* Type-C feruloyl esterase. The effects were an increase in the bread volume, an improvement in the softness of the crumb and the colour of the crust. The effect of different doses of xylanase D222P was also investigated and show a general product improvement in texture, softness and oven-spring with the best result obtain with 0.1 g xylanase per kilogram of flour.

In order to determine further the effect of TsFAEC, baking trials were performed in the presence of a range of TsFAEC units from 6.5 to 65 000 mU determined against MFA in parallel with two controls (addition of xylanase only and no addition of enzyme). Figure 8-1 shows the characteristics of the baked bread and Table 8-1 summarises the observations and measurements performed on the resulting loaves.

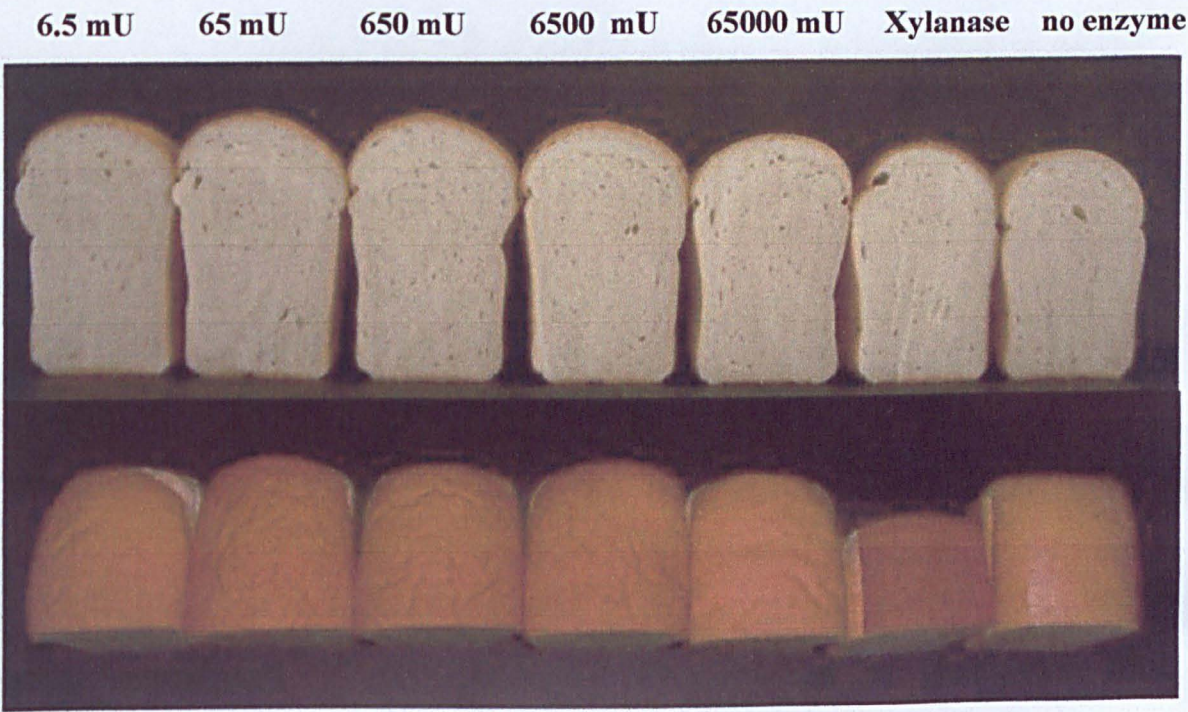


Figure 8-1: Baking trials were performed in the presence of a range of TsFAEC units (6.5 to 65 000 mU) in parallel with two controls, 0.1 g xylanase D222P alone and no addition of enzyme (baking trials from Biocatalysts Limited).

Table 8-1: Measurements of the oven-spring and texture analysis performed on the bread presented in Figure 8-1 (data from Biocatalysts Limited).

Test	Oven-spring (cm)	Texture analysis pressure (g)
No enzyme	25	206
FAE C (65 000 mU)	30	182
FAE C (6 500 mU)	32	143
FAE C (650 mU)	32	184
FAE C (65 mU)	33	165
FAE C (6.5 mU)	33	145
Xylanase D222P (0.1 g)	26	184

In general, the finger-softness, the texture and the colour of the crust were improved in presence of TsFAEC compared to the controls. Oven-spring, was improved in presence of low levels of TsFAEC, whereas higher levels reduced the oven-spring. This could be explained by the generation of higher levels of ferulic acid in these doughs. It is hypothesised that at high concentrations, ferulic acid preferentially forms di-ferulic acid and hence is not available for the reaction with ferulic acid bound pentosan, and by this reaction prevents pentosan aggregation and the increase in dough extensibility (Wang *et al.*, 2002). As a result, formation of di-ferulic acid decreases dough extensibility as can observed with high levels of TsFAEC.

8.4. CONCLUSIONS

Preliminary baking trials have revealed a positive effect for feruloyl esterase in bread making, which is one of the most promising applications for feruloyl esterases. However, the broad substrate specificity exhibited by TsFAEC is likely to be the source of other applications such as carrot juice extraction, alteration of arabinoxylan and pectin gel viscosity for both food and non-food applications, such as wound treatment. Ferulic acid extraction from waste agro-materials for bio-conversion into vanillin or utilization as active ingredient in many skin lotion and sunscreens designed for photo-protection as well as antioxidant in food and non-food industries are also potentially useful applications.

Commercial research is still ongoing and steps to produce a commercially viable new enzyme system with a large range of applications have formed the basis for a patent application filed in June 2002. The patent describes the production and industrial use for the *Talaromyces stipitatus* FAEC feruloyl esterase.

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C.B. Faulds, **Valerie F. Crepin,** I.F. Connerton, J.A. Hermoso and N. Juge. Poster presented at the 4th Carbohydrate Bioengineering meeting (CBM4), 10-13 June 2001, Stockholm - Sweden.

A non-modular type B feruloyl esterase from *Neurospora crassa* exhibits concentration-dependent substrate inhibition

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Feruloyl esterases, a subclass of the carboxylic acid esterases (EC 3.1.1.1), are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in the plant cell wall. The enzymes have been classified as type A or type B, based on their substrate specificity for aromatic moieties. We show that *Neurospora crassa* has the ability to produce multiple ferulic acid esterase activities depending upon the length of fermentation with either sugar beet pulp or wheat bran substrates. A gene identified on the basis of its expression on sugar beet pulp has been cloned and overexpressed in *Pichia pastoris*. The gene encodes a single-domain ferulic acid esterase, which represents the first report of a non-modular type B enzyme (*fae-1* gene;

GenBank accession no. AJ293029). The purified recombinant protein has been shown to exhibit concentration-dependent substrate inhibition (K_m 0.048 mM, K_i 2.5 mM and V_{max} 8.2 units/mg against methyl 3,4-dihydroxycinnamate). The kinetic behaviour of the non-modular enzyme is discussed in terms of the diversity in the roles of the feruloyl esterases in the mobilization of plant cell wall materials and their respective modes of action.

Key words: enzyme, fungus, *Neurospora*, plant cell wall, protein expression, substrate inhibition.

INTRODUCTION

The plant cell wall is a complex architecture of polysaccharides. For the complete hydrolysis of these polysaccharides, micro-organisms require a battery of enzymes. Many plant cell walls contain phenolic acid residues that are ester-linked to the polysaccharide network. In grasses these phenolic compounds are mainly found esterified to arabinoxylans (5-*O*-feruloyl group). In dicotyledons, such as spinach and sugar beet, ferulic acid is esterified to the O-2 or O-3 position of arabinose and to O-6 position of galactose residues in pectin [1,2]. Cross-linking of ferulic acids to cell wall components influences the properties of the cell wall, such as extensibility, plasticity and digestibility. Feruloyl esterases (FAEs; also known as ferulic acid esterases, cinnamoyl esterases and cinnamic acid hydrolases; EC 3.1.1.73), a subclass of the carboxylic acid esterases (EC 3.1.1.1), are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in the plant cell wall [3]. The esterases act to enable and facilitate the access of hydrolases to the backbone wall polymers. Most of the FAEs have been shown to act synergistically with xylanases, cellulases and pectinases to break down complex plant cell wall carbohydrates [4,5]. Several members of the enzyme group have been purified and characterized from aerobic and anaerobic microbes that utilize plant cell wall carbohydrates [6–20]. These enzymes have been classified as either type A or type B, depending on their substrate specificity for aromatic moieties. The enzymes show a preference for the phenolic linkage to the primary sugar, and vary in their ability to release dehydrodiferulic acids from esterified substrates. Re-

garding specificity against synthetic substrates, type A FAEs are active against methyl ferulate (MFA), methyl sinapate (MSA) and methyl *p*-coumarate (MpCA), but not methyl caffeate (MCA), whereas type B are active against MCA, MFA and MpCA, but not MSA. Only type A FAEs are able to hydrolyse synthetic ferulate dehydrodimers and to release dimers from agro-industrial materials, as they have a preference for more hydrophobic substrates with bulky substituents on the benzene ring [21–23].

Esterases are novel enzymes with considerable potential for agri-food processing applications. For example, phenolic acids derived from plant cell walls have long been used as food preservatives to inhibit microbial growth. *Aspergillus niger* has been shown to transform ferulic acid into vanillic acid and, similarly, *Pycnopotus cinnabarinus* converts ferulic acid into vanillin, an essential flavour in the food industry. Ferulic acid is also an effective natural antioxidant with potential applications in the pharmaceutical and food industries [3,24–28]. There is therefore considerable scope to utilize FAEs to produce and exploit natural products that can be extracted from otherwise waste agri-food material.

The two major FAEs of *Aspergillus niger* have been the focus of several studies over the last decade. However, in recent years, our knowledge of the FAE family has expanded, with reports of new enzyme activities, the characterization of gene sequences and the first crystal structure [15–19,29]. Molecular analysis of FAE genes and their predicted protein sequences has revealed that many of these enzymes are modular, comprising a catalytic domain translationally fused to a non-catalytic cellulose-binding

Abbreviations used: Ara₂F, [2-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1,5)-L-arabinofuranose; CBD, cellulose-binding domain; FAE, feruloyl esterase (ferulic acid esterase; cinnamoyl esterase; cinnamic acid hydrolase; EC 3.1.1.72); *Fae-1*, feruloyl esterase from *Neurospora crassa*; MCA, methyl caffeate (methyl 3,4-dihydroxycinnamate); MFA, methyl ferulate (methyl 4-hydroxy-3-methoxycinnamate); MpCA, methyl *p*-coumarate (methyl 4-hydroxycinnamate); MSA, methyl sinapate (methyl 3,5-dimethoxy-4-hydroxycinnamate); ORF, open reading frame; SBP, sugar beet pulp; WB, wheat bran.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSSD Nucleotide Sequence Databases under accession number AJ293029.

domain (CBD) [15,16,19,30], or are produced as monomeric enzyme units pre-designated to be assembled into longer cohesive units, such as the cellulosome of *Clostridium* [31].

In the present paper, we report the ability of the filamentous fungus *Neurospora crassa* to produce alternative FAE activities in response to the availability of plant cell wall carbohydrates, and the identification of a gene (*fae-1*) specifically induced on sugar beet pulp (SBP) that encodes the first example of a non-modular type B FAE (Fae-1) to be reported. The *N. crassa* gene has been overexpressed in *Pichia pastoris* [32] and the purified recombinant protein was shown to exhibit concentration-dependent substrate inhibition. The kinetic behaviour of this non-modular enzyme is discussed in terms of the structural diversity of the FAEs and their respective catalytic roles in the mobilization of plant cell wall materials.

EXPERIMENTAL

Materials

The methyl esters of ferulic acid, caffeic acid, *p*-coumaric acid and sinapic acid were obtained from Apin Chemicals Ltd (Abingdon, Oxon, U.K.). The corresponding free acids, chlorogenic acid, naphthyl acetate and all other standard chemicals were obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, U.K.).

Strains, vectors and media

Escherichia coli TOP10 (Invitrogen) was used as the bacterial host for DNA manipulations with the pCR®2.1-TOPO® cloning vector. *E. coli* TAP90 was used as the host for lambda [33] and *E. coli* TG1recF⁻ was used for propagation of plasmids and as a recipient in transformation. The vector pEMBL18 [34] was used for subcloning the putative *fae-1* gene. *Pichia pastoris* (*his4*) GS115 was used to produce recombinant Fae-1 with the expression vector pPIC3.5K (Invitrogen). *Neurospora crassa* wild-type ST A (74 A) was maintained on minimal Vogel's agar (1.5%, w/v) plus 2% (w/v) sucrose [35]. Liquid cultures were grown in minimal Vogel's supplemented with 2% (w/v) carbon sources [sucrose, SBP or wheat bran (WB)].

Detection of esterase activities in *Neurospora crassa* culture supernatants

N. crassa mycelia were grown in 100 ml of Vogel's sucrose medium for 2 days at 30 °C on a flat-bed shaker (200 rev./min). Cultures were vacuum-filtered through a sterile Buchner funnel, washed with distilled water and transferred aseptically to 500 ml of Vogel's supplemented with 2% (w/v) SBP or 2% (w/v) WB. Samples of 5 ml of supernatant were removed every 24 h for a period of 9 days. FAE activities in supernatant samples were assayed spectrophotometrically at 335 nm by the method of Ralet and co-workers [1]. Activity was measured against MFA (methyl 4-hydroxy-3-methoxycinnamate), MCA (methyl 3,4-dihydroxycinnamate), MSA (methyl 3,5-dimethoxy-4-hydroxycinnamate) and MpCA (methyl 4-hydroxycinnamate) at 0.1 mM final concentration in 100 mM Mops buffer (pH 6.0), using 20 µl of culture supernatant. Protein concentration was determined by the Bradford method [36] using the Coomassie Protein Assay Reagent from Pierce.

Genomic DNA extraction

DNA was extracted in duplicate experiments using the ethanolic perchlorate method of Stevens and Metzenberg [37], after growth of *Neurospora crassa* on minimal Vogel's sucrose medium.

RNA extraction

Neurospora crassa ST A (74 A) was grown at 30 °C in a shaking incubator (200 rev./min) in 100 ml of minimal Vogel's supplemented with 2% (w/v) sucrose. After 48 h, cultures were vacuum filtered through a sterile Buchner funnel washed with sterile distilled water and transferred aseptically to 100 ml of Vogel's supplemented with the selected carbon source (SBP or WB). After 3–4 days of growth, cultures were filtered through muslin and filtrates (pre-digested medium) were sterilized by autoclaving before inoculation with a fresh mycelial mat. Mycelia were incubated for 6 h to induce FAE expression before harvesting. Total RNAs were extracted in two independent experiments from mycelia following growth on pre-digest supplemented media [Vogel's plus 2% (w/v) SBP or WB] or minimal Vogel's plus 2% (w/v) sucrose, using the method described by Sokolovsky et al. [38]. Poly-adenylated RNAs were selected from total RNAs using oligo(dT) chromatography.

RNAs were extracted every 48 h in independent duplicate experiments during the direct fermentation of SBP and WB by *N. crassa*, as described above for the detection of esterase activities.

Screening of the genomic library

Plaques of the *N. crassa* genomic lambda library λJ1-74A [39] were lifted in duplicate and probed with cDNA synthesized from poly(A)⁺ RNA extracted from mycelia cultured on either sucrose or SBP. Superscript™ (Gibco BRL) was employed to reverse transcribe poly(A)⁺ mRNA using [α -³²P]dCTP (~3000 Ci·mmol⁻¹; Amersham Pharmacia Biotech) to label the cDNAs. The cDNAs were precipitated with 2.5 M ammonium acetate and propan-2-ol, resuspended in water and boiled before use as a hybridization probe against plaque lifts.

Southern and Northern blots

EcoRI restriction digests of *N. crassa* genomic DNA were electrophoresed in 0.8% (w/v) agarose gels and blotted by capillary transfer on to Hybond-N⁺ (Amersham Pharmacia Biotech) nylon membranes before fixation with UV light. The blots were probed with a DNA fragment specific for the *fae-1* gene, labelled using the PCR DIG-labelling system (Boehringer Mannheim) according to the manufacturer's instructions. Colorimetric detection was performed in the presence of Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates, according to the manufacturer's instructions (Boehringer Mannheim).

RNAs were electrophoresed in a formaldehyde denaturing agarose gel according to the method described by Sambrook and colleagues [40] with 1 × Mops, blotted by capillary transfer on to a Hybond-N⁺ nylon membrane and hybridized to a labelled DNA probe specific for the *fae-1* gene. Colorimetric detection was performed as described above.

Overlapping PCR

Overlapping-PCR amplification was used in order to amplify cDNA of the *Neurospora crassa* FAE gene (*fae-1*) from genomic DNA. Two specific primers were designed on either side of a putative intron (ssN1F and I1R primers 5' of the intron, and I1F and C1R primers 3' of the intron) (Table 1). The reverse primer (I1R) has a tail, the sequence of which complements the forward primer (I1F), and vice versa. Two PCR reactions were performed to amplify separately the sequences upstream and downstream of the intron using the primer combinations ssN1F/I1R and I1F/C1R. DNA amplification was carried out through 30 cycles

Table 1 Names and sequences of oligonucleotides used to amplify the *N. crassa fae-1* cDNA

Specific restriction sites were incorporated into the PCR primer sequences: *Bam*HI at the 5' end of the ssN1F primer and *Bam*HI–*Not*I at the 5' end of the C1R primer (in bold). † indicates the stop codon.

Primer name	Primer sequences with their translations															
ssN1F	5'-AAAAGGATCCAT	ATG	TTG	CCC	AGA	ACA	TTG	C-3'								
		M	L	P	R	T	L	L								
C1R	5'-AAAAGGATCCGCGGCCG	CGA	CAT	CTA	GTT	GAT	CAA	CCC-3'								
				†	N	I	L	G								
IIF	5'-C	GCC	ATT	ATT	GTG	GCT	CTC	CAC	GGC	TGT	GGC	GG-3'				
		A	I	I	V	A	L	H	G	C	G					
IIR	5'-GCC	ACA	GCC	GTG	GAG	AGC	CAC	AAT	AAT	GGC	GGG	C-3'				
	G	C	G	H	L	A	V	I	I	A	P					

of denaturation (30 s at 94 °C), annealing (1 min at 60 °C) and extension (1 min at 72 °C). Amplified fragments were gel-purified and equal quantities of each were mixed together. The DNA mixture was denatured at 100 °C for 10 min and the reaction was allowed to cool down slowly at room temperature. dNTPs (0.5 µl, 25 mM) (Promega), 10 × Klenow buffer (2 µl), Klenow enzyme (1 µl; 2 units/µl) (Boehringer Mannheim) and water were added to make the reaction up to 20 µl (final volume). The reaction mixture was incubated at 37 °C overnight to allow synthesis of double-stranded DNA. DNA was precipitated with 0.1 vol. of 3 M sodium acetate, pH 5.2, and 2 vol. of 100 % (v/v) ethanol, recovered in water and amplified by PCR with primers designed on the 5' and 3' ends of the *fae-1* cDNA (ssN1F and C1R; Table 1). DNA amplification was carried out through 30 cycles of denaturation (30 s at 94 °C), annealing (1 min at 60 °C), and extension (1 min at 72 °C). The resulting PCR product was cloned into the pCR®2.1-TOPO® vector (TOPO TA Cloning® kit from Invitrogen) and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

Construction of expression vector

The cDNA fragment (879 bp) containing the *N. crassa fae-1* native signal sequence was isolated from the cloning vector by restriction endonuclease digestion with *Bam*HI–*Not*I and ligated into *Bam*HI–*Not*I-digested pPIC3.5K expression vector.

Transformation of *P. pastoris* and selection of secreting transformants

The transformation of *Pichia pastoris* GS115 was performed using the electroporation method [41]. For transformation, 1–5 µg of vector with or without insert (pPIC3.5K/*fae-1* and pPIC3.5K respectively) was linearized with *Dra*I restriction enzyme to allow gene replacement at the *Pichia pastoris AOX1* gene. Transformants were selected by their ability to grow at 30 °C on histidine-deficient regeneration medium (RDB: Regeneration Dextrose Base; Invitrogen). To assess the His⁺ transformants for methanol utilization (Mut) phenotype, colonies were picked and patched on to minimal methanol plates and minimal dextrose plates (Invitrogen) in a regular pattern. After 5 days at 30 °C, normal growth on minimal dextrose plates and slow growth on minimal methanol plates is indicative of the Mut⁺ (Mut-sensitive) phenotype. Mut⁺ colonies were used to inoculate 10 ml of buffered complex glycerol medium, pH 6.0 (Invitrogen) and incubated at 30 °C with vigorous shaking (> 200 rev./min) until $D_{600} = 10$ –20 (2 days). Cells were harvested by centrifugation at 4000 g for 10 min at room

temperature and resuspended in 2 ml of buffered complex methanol medium, pH 6.0 (Invitrogen) and incubated at 30 °C with vigorous shaking for 3 days. Methanol (100 %) was added to a final concentration of 0.5 % methanol every 24 h to maintain induction. After 3 days of induction, cells were then pelleted by centrifugation at 13000 g for 3 min at room temperature and supernatants were analysed with SDS/PAGE [12 % (w/v) bisacrylamide] for protein expression [40]. To confirm that the positive clones were FAE-producing transformants, culture supernatants were assayed for activity against MCA and MFA using reverse-phase HPLC [6].

The N-terminal amino acid sequence of the recombinant protein was determined after separation by SDS/PAGE (12 % bisacrylamide) and Western transfer on to a PVDF membrane (Boehringer Mannheim). The membrane was stained with Coomassie Blue R250 and the candidate band was extracted followed by N-terminal amino acid sequencing using a Sequencer Model 473A (Applied Biosystems).

Purification of recombinant *N. crassa Fae-1* expressed in *P. pastoris*

Large-scale culture was performed in 2-litre Erlenmeyer flasks. Cells were grown in 500 ml of buffered complex glycerol medium, pH 6.0, at 30 °C for 48 h (D_{600} 20–25). The recovered cells were resuspended in 200 ml of buffered complex methanol medium, pH 6.0, and grown for a further 5 days at 30 °C (200 rev./min). The culture was centrifuged at 13000 g for 15 min at room temperature and 196 ml of supernatant was recovered. Solid ammonium sulphate was added to 1 M and clarified by centrifugation at 10000 g for 15 min at 4 °C. Aliquots of 30 ml were loaded on to a butyl-Sepharose 4 Fast Flow hydrophobic interaction chromatography column (2.6 cm × 10 cm) membrane (Amersham Pharmacia Biotech). Unbound material was eluted (5 ml · min^{−1}) with 50 mM sodium phosphate buffer, pH 7.0, 1.0 M ammonium sulphate (buffer A), followed by elution of the bound proteins with a gradient of buffer A/buffer B (50 mM sodium phosphate buffer, pH 7.0). Fractions were collected and assayed for activity against MCA using the spectrophotometric method [1]. Active fractions were pooled and desalted through a NAP-5 column membrane (Amersham Pharmacia Biotech) into water and a 240–400 nm absorbance spectrum was recorded. The molar absorption coefficient for Fae-1 was determined from its amino acid content, and this value (51 790 M^{−1} · cm^{−1}) was used to determine the concentration of the sample, using the Beer–Lambert law.

Fae-1 was concentrated 13-fold through a 10000 Da cut-off ultrafiltration membrane (Amicon), and 3 ml of sample was dialysed against 5 litres of MilliQ water using a Slide-A-Lyzer

cassette (10000 Da cut-off; Pierce Chemical Co.). Electrospray ionization-MS of Fae-1 was performed using a Quattro II instrument (Micromass, Manchester, U.K.).

Enzyme assays

Assays for FAE activity were performed by incubating methyl esterified substrates (MFA, MCA, MSA or MpCA) [6] or the feruloylated oligosaccharide Ara₂F {[2-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1,5)-L-arabinofuranose} [42], in a final volume of 0.5 ml for 15 min in 100 mM Mops buffer, pH 6.0, at 37 °C. Agro-industrial waste such as SBP, WB or brewer's (spent) grain (10 mg) was incubated in the presence of FAE for 3 h in 100 mM Mops buffer, pH 6.0, at 37 °C. Reactions were terminated by the addition of acetic acid (0.2 ml), and 0.2 ml samples were assayed for phenolic acids by reverse-phase HPLC [11]. One unit of activity is defined as the amount of enzyme (mg) or ml of culture supernatant releasing 1 μ mol of free ferulic acid per min under the defined conditions.

Kinetic constants (k_{cat} , K_m , V_{max}) were calculated from initial-rate data using the Michaelis-Menten equation: $v = (V_{max}[S])/(K_m + [S])$. Due to substrate inhibition, the kinetic constants were determined at low values of substrate (from 0.02 mM to 0.4 mM), which is permissible when K_i is large compared with K_m . The K_i was estimated from initial-rate data at various substrate concentrations (from 0.02 mM to 3 mM), using the equation with respect to a substrate inhibition: $v = (V_{max}[S])/(K_m + [S] + ([S]^2/K_i))$. Acetylcholinesterase activity was determined using α -naphthyl acetate (0–2.5 mM) in a 1 ml reaction volume in 100 mM sodium phosphate buffer, pH 6.0, at 37 °C, by following the increase in absorbance at 235 nm over 5 min. Acetylcholinesterase activity was calculated from the initial rate of change of A_{235} using a molar absorption coefficient for α -naphthyl acetate of 24000 M⁻¹ cm⁻¹ [43]. One unit of activity is defined as the amount of enzyme forming 1 μ mol of product per min at pH 6.0 and 37 °C.

Temperature-activity and pH-activity profiles

To determine the pH optimum, recombinant Fae-1 was incubated for 15 min at 37 °C with MFA (0.1 mM final concentration in a 500 μ l reaction) in McIlvaine's buffer titrated to a pH within the range 3.0–7.5, and in phosphate buffer titrated to a pH within the range 6.0–8.0. To determine the temperature optimum, recombinant Fae-1 was incubated for 15 min with MFA (0.1 mM final concentration in a 500 μ l reaction) in 100 mM Mops, pH 6.0, at temperatures ranging from 20 °C to 80 °C. The temperature stability of the recombinant Fae-1 was determined at 60 °C. The enzyme was incubated for up to 60 min at 60 °C, and aliquots were removed every 10 min and assayed for activity against MFA (0.1 mM final concentration in a 500 μ l reaction), for 15 min in 100 mM Mops, pH 6.0, at 37 °C. The reactions were stopped by the addition of 0.2 ml of acetic acid. Controls containing the reaction mixture plus acetic acid were incubated to eliminate interference. Samples and blanks were centrifuged at 13000 g for 5 min prior to HPLC analysis of the released ferulic acid.

RESULTS

FAE activity in the culture supernatant

Neurospora crassa ST A (74 A) was grown in minimal Vogel's medium supplemented with 2% (w/v) sucrose for 24 h, then transferred to Vogel's containing 2% (w/v) SBP or WB for 9 days. Culture supernatant samples were removed every 24 h and assayed for FAE activities against the methyl esters of caffeic

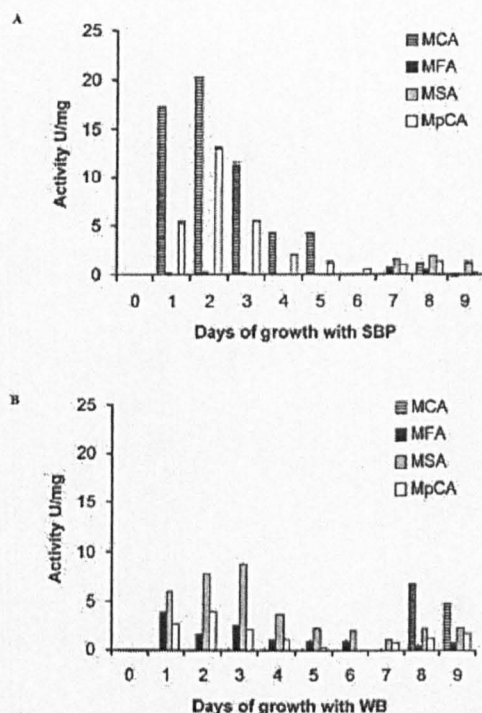


Figure 1 FAE activities detected in culture supernatants of *Neurospora crassa*

FAE activity (units/mg) was measured in culture supernatants of *Neurospora crassa* grown with SBP (A) or WB (B). FAE activities were measured with MCA, MFA, MSA and MpCA as a function of incubation time (0–9 days). Day 0 corresponds to the supernatant sample analysed after 24 h of growth with Vogel's medium plus 2% (w/v) sucrose.

(MCA), sinapic (MSA), ferulic (MFA) and *p*-coumaric (MpCA) acids (Figure 1). FAE activity was detected following growth on SBP (A) and WB (B). The substrate activity profile, however, suggested the presence of two FAE activities. Samples removed in the first 3 days of growth on WB showed FAE activity against MFA, MSA and MpCA, but not MCA, whereas samples removed during the first 3 days of growth on SBP showed FAE activity mainly against MFA, MCA and MpCA, but not MSA. As FAEs are discriminated based on their ability to utilize MSA and diferulates [12,16,19–21,23], we can identify type A and type B FAE activities from *N. crassa* grown on WB or SBP. In addition, at days 7–8 and 8–9 of growth on SBP and WB respectively, FAE activity was detected against all four synthetic monomeric substrates. These data confirm that *N. crassa* is able to induce and adapt the production of FAEs according to the carbon source present in the culture medium, and as a function of growth time. Therefore *N. crassa* is a potential source of new FAEs.

Genomic library screen

Labelled cDNA reverse-transcribed from mRNA extracted from mycelia of *Neurospora crassa* grown on either sucrose or SBP was used to probe duplicate plaque lifts of a genomic library in order to identify genes differentially expressed on SBP, in particular the genes encoding FAEs [44]. The library screen revealed several positives clones. Restriction digests of the cloned DNAs were transferred to a Southern blot and hybridized with

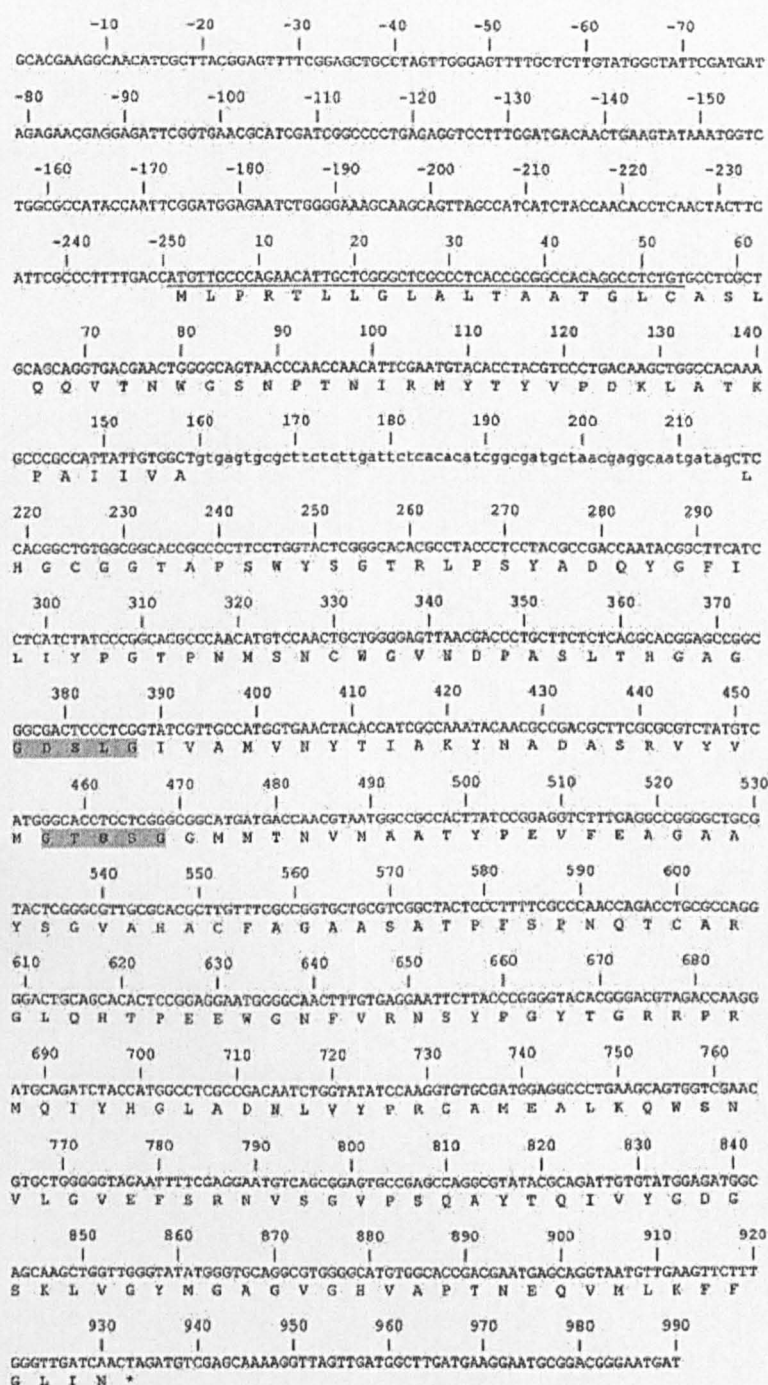


Figure 2 Nucleotide and deduced amino acid sequences of the *fae-1* gene from *Neurospora crassa*

The signal peptide is underlined. The 56 bp intron is shown in lower case. The two sites containing the consensus motif (G-X-S-X-G) for the active-site serine are shown by grey boxes, with the putative active-serine in bold. The stop codon is indicated by *.

the reverse-transcribed cDNA to identify the fragments containing the genes of interest, which were in turn subcloned into the pEMBL18 vector and sequenced. The translation of one of these clones showed 50 % identity with a type B FAE from *Pe. funiculosum* [19] and 45 % identity with an acetylsterase reported

from *Aspergillus awamori* [45]. Comparative analysis of the sequence indicated that a reading frame of 876 bp was present, but interrupted by a single intron. A cDNA for this gene was constructed by overlapping PCR from *N. crassa* genomic DNA. Specific primers were designed based on the genomic sequence in

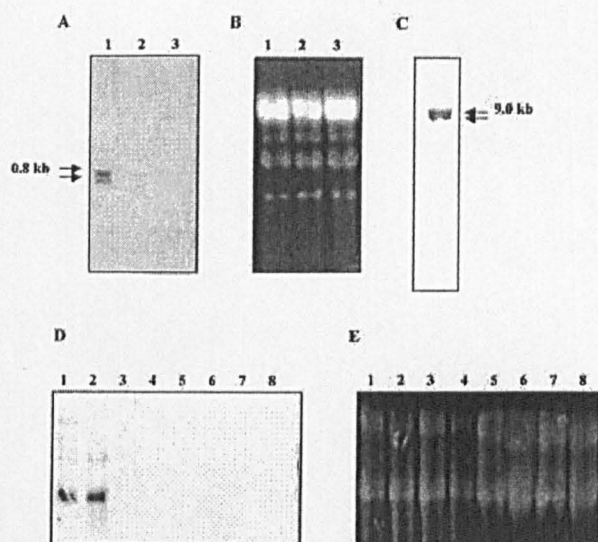


Figure 3 Northern and Southern blots

(A) Northern blot of RNAs extracted from *N. crassa* following 6 h of growth on predigested selective SBP (lane 1) or WB (lane 2) media, and following 48 h of growth on minimal Vogel's medium plus 2% (w/v) sucrose (lane 3). The membrane was hybridized with the *N. crassa* genomic clone coding for a type B FAE. (B) Ethidium bromide stain of the membrane shown in (A), demonstrating equal loading of the RNA samples. (C) Southern blot of *N. crassa* genomic DNA digested with the restriction enzyme *EcoRI*, known to cut once in the genomic clone. The relative molecular sizes of the main hybridization products are recorded alongside the blots. (D) Northern blot of RNAs extracted from *N. crassa* following 2, 4, 6 and 8 days of fermentation with 2% (w/v) SBP (lanes 1–4 respectively) or 2% (w/v) WB (lanes 5–8 respectively). (E) Ethidium bromide stain of the membrane shown in (D) demonstrating equal loading of the RNA samples. Southern and Northern blots were repeated using independent DNA and RNA preparations, as indicated in Experimental section.

order to independently amplify the sequences upstream and downstream of the intron that were joined as overlapping PCR fragments. After cloning in the pCR®2.1-TOPO® vector, the nucleotide sequence resulting from the overlapping-PCR products was sequenced. The open reading frame (ORF) encoded a protein of 292 amino acids, including an 18-amino-acid signal peptide (Figure 2). The calculated average mass of the mature protein is 29286 Da, and the theoretical pI is 8.26. Inspection of the protein sequence reveals the presence of two motifs characteristic of the serine esterase family (Gly-Xaa-Ser-Xaa-Gly) [19,46,47]. The first motif (Gly-Asp-Ser-Leu-Gly) is centred on serine-91, but is not conserved within the *Pe. funiculosus* cinnamoyl esterase sequence or the acylesterase sequence from *Aspergillus awamori*. The second motif (Gly-Thr-Ser-Ser-Gly), centred on serine-118, is conserved in the *Pe. funiculosus* cinnamoyl esterase and the *Aspergillus awamori* acylesterase sequences, as well as in other members of the serine esterase family [19]. Serine-118 is therefore a candidate for a component of the catalytic triad featured in all the family members. The translated sequence, however, does not feature a recognizable CBD, as has been observed with the type B FAE reported from *Pe. funiculosus* [19].

Northern and Southern blots

To confirm the differential expression of the putative FAE gene, RNAs were extracted after growth on SBP, WB or sucrose and analysed by Northern blot (Figures 3A and 3B). Two transcripts

specific to growth on SBP were detected by hybridization with a DNA probe. These data are consistent with the expression profile of type B FAEs observed in other fungal species [12,19,20]. In order to determine whether the two transcripts arise from two independent genes or are the product of a single gene, a Southern blot of genomic DNA was performed (Figure 3C). *N. crassa* genomic DNA was digested with *EcoRI* (known to cut once in the genomic sequence) and hybridized with the genomic sequence to produce two bands, as might be anticipated for a single-copy gene. The transcripts are therefore likely to be products of the same gene. The mRNA expression of the putative FAE gene was also analysed by Northern blot at 48 h intervals over the period of fermentation with raw particulate SBP and WB substrates that correspond to the detection of the esterase activities in the culture supernatants (Figures 3D and 3E). The putative type B FAE mRNA was expressed during the early stages of growth on SBP, and its expression decreased later in the fermentation, in parallel with the type B enzyme activity (Figure 1A). During growth on WB, expression of the mRNA was barely detectable throughout the course of the fermentation (Figure 1B).

Overexpression in *Pichia pastoris*

In order to confirm the function of the putative gene product and to provide a ready source of the enzyme for study, the *N. crassa* cDNA was overexpressed in *Pichia pastoris*. The *P. pastoris* system was selected as it had already been proven to efficiently secrete active FAE [32]. The cDNA of the FAE from *N. crassa*, containing the native signal sequence, was cloned into the expression vector pPIC3.5K under the control of the alcohol oxidase 1 gene promoter (*AOX1*). The resulting expression plasmid (pPIC3.5K/*fae-1*) and the parent vector were linearized with *DraI* restriction enzyme, allowing gene replacement at *AOX1*, and used to transform *P. pastoris*. Both transformations generated hundreds of His⁺ transformants. The His⁺ transformants were scored for their ability to grow on minimal methanol media. A total of 13 Mut⁺ pPIC3.5K/*fae-1* transformants were screened for expression in small-scale cultures along with two colonies transformed with the parental vector as a control for background protein secretion levels. Culture supernatants were analysed by SDS/PAGE for secreted protein products. Five pPIC3.5K/*fae-1* transformants produced a major secreted protein band of approx. 35 kDa. No protein was detected with the vector controls. To confirm that these cultures were FAE-producing transformants, culture supernatants were assayed for activity against MCA, MFA and MSA. The recombinant protein was found to be active as a FAE, and showed the characteristics of a type B FAE, in that it was inactive against MSA. We therefore have named the *N. crassa* protein Fae-1, as the product of the *fae-1* gene (GenBank® accession number AJ293029). The most active clone (clone 10) was retained for large-scale expression in order to purify and characterize the recombinant enzyme (Figure 4A).

The recombinant Fae-1 was transferred by electroblotting to a PVDF membrane for protein sequence analysis. The first 10 amino acids of the N-terminus of the expressed protein were determined to be Ala-Ser-Leu-Gln-Gln-Val-Thr-Asn-Trp-Gly. This sequence is identical to the sequence predicted for the mature Fae-1 product, indicating that *P. pastoris* was able to efficiently process the *N. crassa* native signal sequence. Four N-linked glycosylation sites can be predicted from the Fae-1 sequence. Glycosylation of the *P. pastoris* product was confirmed by electrospray ionization-MS, which explained the mass difference of 6 kDa between the calculated average mass of the mature protein and that estimated from SDS/PAGE of the

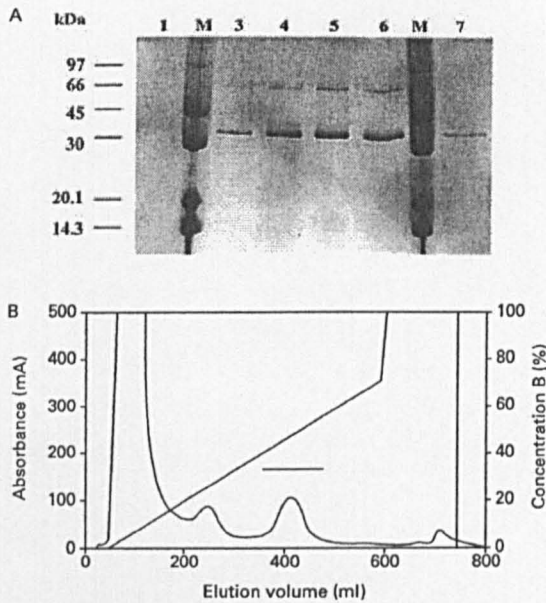


Figure 4 Purification of recombinant Fae-1

(A) SDS/PAGE on 12% (w/v) acrylamide. M, standard protein molecular mass markers. Lane 1, negative control; lanes 3–6, samples of culture supernatant removed daily (days 2–5 respectively) from a large-scale culture of *P. pastoris* transformant expressing recombinant Fae-1 (clone 10); lane 7, recombinant Fae-1 following purification. (B) Elution profile of recombinant *N. crassa* Fae-1 on a butyl-Sepharose Fast Flow 4 hydrophobic interaction chromatography column. The fractions active against MFA are marked with a horizontal line, eluting around 400 ml.

recombinant Fae-1. Six main peaks were apparent on MS, ranging between 34716 and 35523 Da. The main mass was calculated to be 35040 Da. The other peaks correspond to

different levels of glycosylation of the enzyme, where each peak corresponds to the addition of one sugar unit. The unglycosylated protein has a calculated molecular mass of 29286 Da, and thus the main product can be estimated to contain 32 mannose residues.

Purification and characterization of recombinant Fae-1

The pPIC3.5K/*fae-1* *Pichia* transformant (clone 10) was grown for 5 days in buffered complex methanol medium at 30 °C before harvesting. Aliquots of 30 ml of the culture supernatant were fractionated on a butyl-Sepharose Fast Flow 4 hydrophobic interaction chromatographic column. A single peak of activity against MCA was eluted (Figure 4B), and active fractions were pooled. The enzyme yield after purification was determined to be 73%. The protein concentration of the recombinant Fae-1 was calculated from the absorbance of the desalted sample at 280 nm. The molar absorption coefficient of Fae-1 ($51\,790\text{ M}^{-1}\cdot\text{cm}^{-1}$) was determined from the amino acid sequence and the concentration of the purified esterase, determined to be 0.034 mg of protein/ml of sample. The yield of secreted recombinant Fae-1 protein from methanol-induced shake-flask culture was $210\text{ mg}\cdot\text{l}^{-1}$ after 5 days.

Table 2 presents the kinetic constants (V_{\max} , k_{cat} , K_m and k_{cat}/K_m) calculated from the initial-rate activity of recombinant *N. crassa* Fae-1 against hydroxycinnamic acid esters. Substrate concentrations between 0.02 mM and 0.4 mM were employed to estimate the kinetic constants from the Michaelis–Menten equation. As expected of a type B FAE, Fae-1 showed a high catalytic efficiency for the hydrolysis of MCA and MpCA, but no activity was detected against MSA. MCA and MpCA substrates were turned over at higher rates, but the catalytic efficiency with MFA was lower. The low rate recorded for the Ara₂F substrate is consistent with a requirement for a longer feruloylated oligosaccharide to produce optimal hydrolysis. The catalytic efficiency of *N. crassa* Fae-1 for the hydrolysis of Ara₂F ($12.87\text{ mM}^{-1}\cdot\text{s}^{-1}$) is 112-fold greater than that recorded for the

Table 2 Substrate specificity of *Neurospora crassa* Fae-1 with synthetic (A) and natural (B) substrates

Nd, not detected. FAEA is type A FAE from *A. niger*. Released metabolites are expressed as a percentage of total alkali-extractable phenolic acids, after 3 h of incubation with 100 m-units of Fae-1.

(A)				
Substrate	V_{\max} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{mM}^{-1}\cdot\text{s}^{-1}$)
Methyl esters				
MFA	8.97	5.24	0.25	21
MpCA	20.87	12.19	0.021	580
MCA	8.20	4.80	0.02	100
MSA	Nd	Nd	Nd	Nd
Feruloylated oligosaccharides				
Ara ₂ F	10.34	6.04	0.46	13
Acetylated substrates				
α -Naphthyl acetate	6.65 (Fae-1)/11.64 (FAEA)	3.44 (Fae-1)/5.86 (FAEA)	1.13 (Fae-1)/5.45 (FAEA)	3 (Fae-1)/1 (FAEA)
Chlorogenated substrates				
Chorogenic acid	9.14	5.34	0.18	29
(B)				
Substrate	Released metabolite (%)			
	Ferulic acid	<i>p</i> -Coumaric acid	Dimers	
WB	1.2	0.7	Nd	
Spent grain	2	0.7	Nd	
SBP	2.5	0	Nd	

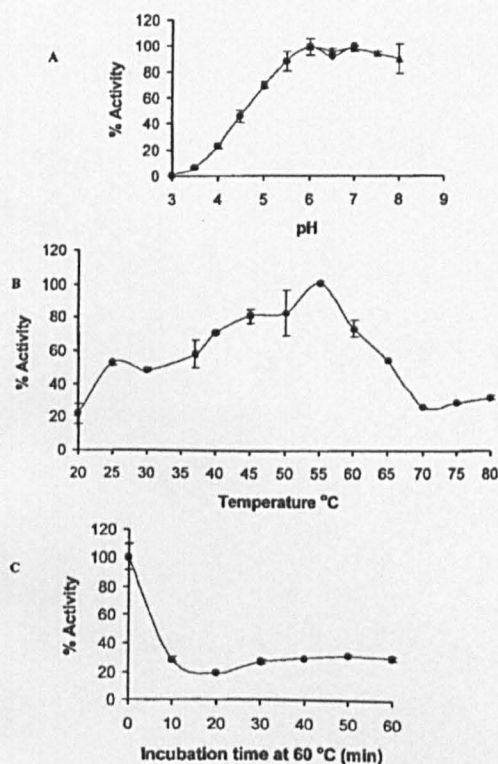


Figure 5 Temperature- and pH-activity profiles of recombinant Fae-1
(A) pH profile of recombinant Fae-1. Activities were determined against MFA (0.1 mM) over the pH ranges 3.0–7.0 using Mclvaine's buffer (●) and pH 6.0–8.0 using a phosphate buffer (▲). (B) Temperature profile of recombinant Fae-1 determined with MFA (0.1 mM) between 20 and 80 °C (5 °C increments). (C) Temperature stability of recombinant Fae-1. The temperature stability was estimated at 60 °C over 60 min. Samples were removed every 10 min and assayed for residual activity at 37 °C against MFA (0.1 mM).

A. niger cinnamic acid esterase CinnAE or the *Pir. equi* cinnamoyl ester hydrolase CEH (EstA') [5,16,42]. However, the rate of turnover of Ara₆F by Fae-1 is 40-fold lower than the catalytic efficiency of *Pe. funiculosus* FAEB (530 mM⁻¹ · s⁻¹) [19].

Fae-1 was also tested for its ability to release ferulic acid and *p*-coumaric acid from cereal-derived material. Fae-1 was shown to release ferulic acid from spent brewer's grain at a level similar to that reported for type A FAE from *A. niger* (Table 2). Fae-1, however, did not release ferulated dimers from any of the agro-industrial materials tested, which is consistent with the properties of other type B FAEs [12,19]. The acetylsterase activity of Fae-1 was investigated using α -naphthyl acetate as substrate. Fae-1 is able to efficiently hydrolyse α -naphthyl acetate with an activity in excess of that measured for FAEA from *A. niger* [48], but lower than the specificity of true acetylxyln esterases (Table 2). In addition, there are probably constraints on the access of the FAEs compared with the xylan esterases, as neither FAEA from *A. niger* nor Fae-1 can release acetate from cereal cell wall material.

The pH optimum of recombinant Fae-1 was determined against MFA for a range of pH values between 3.0 and 8.0 (Figure 5A). Maximum activity was recorded at pH 6.0, with enzyme activity stable between pH 6.0 and 7.5. The enzyme was generally compromised at acidic pH. The temperature optimum of recombinant Fae-1 was determined against MFA for a range of

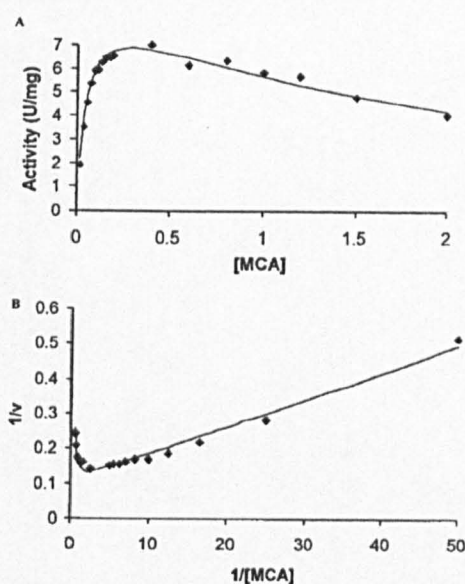


Figure 6 Fae-1 activity profile with MCA using a range of substrate concentrations between 0.02 and 2 mM
(A) Substrate concentration against activity (units/mg). (B) Double-reciprocal plot of substrate concentration against activity (units/ml).

Table 3 Kinetic constants for Fae-1 determined against synthetic methyl esters

Substrate	K_m (mM)	K_i (mM)	χ^2
MFA	0.25	2.82	0.067
MpCA	0.021	2	0.082
MCA	0.048	2.5	0.003
MSA	Nd	Nd	Nd

temperatures between 20 and 80 °C (Figure 5B). The recombinant enzyme had a temperature optimum of 55 °C for the hydrolysis of MFA. Above this temperature, the activity dropped significantly, to reach a plateau (24 % activity) between 70 and 80 °C. The temperature stability of recombinant Fae-1 was measured by incubating the enzyme at 60 °C for 60 min. Samples were removed every 10 min and assayed for residual activity against MFA at 37 °C (Figure 5C). After 10 min at 60 °C, the enzyme had lost 80 % of its activity. However, the residual activity was retained even after 60 min at 60 °C.

Substrate inhibition of Fae-1

At high concentrations of the synthetic monomeric substrates (MCA, MFA, MSA and MpCA), the enzyme activity of Fae-1 was shown to fall. The initial-rate data could be related to the rate equation derived for substrate inhibition: $v = (V_{max}[S]) / (K_m + [S] + ([S]^2/K_i))$. This equation was applied to first estimate the kinetic constants V_{max} and K_m , and to determine K_i values at substrate concentrations from 0.02 to 0.3 mM. As the K_i was large compared with K_m , at low concentrations of substrate (between 0.02 and 0.3 mM) the enzyme rates approximated to the Michaelis–Menten equation. Therefore, at low substrate

concentrations, the kinetic constants V_{\max} and K_m were determined in the usual way from the Michaelis–Menten equation (see the Experimental section), using Lineweaver–Burk double-reciprocal plots. The kinetic curves obtained with MCA are shown in Figures 6(A) and 6(B), and kinetic constants determined for all four synthetic substrates are presented in Table 3.

DISCUSSION

We report the development of FAE activities in the utilization of plant cell wall materials by the filamentous mesophilic fungus *Neurospora crassa*. We show that *N. crassa* will produce either type A or type B FAE activity when grown on WB or SBP respectively. The substrate specificity profiles are similar to those reported for the *A. niger* esterases FAEA (type A) and CinnAE (type B) against synthetic methyl esters [11,12,19,49]. During the early stages of the growth period (days 2–5), *N. crassa* produces type A and type B carbon-source-dependent esterase activities; however, later in the fermentation of either WB or SBP (days 5–6), these activities become repressed, to be replaced by the expression of a new activity (days 7–9), characterized by its ability to hydrolyse all four synthetic substrates tested (MCA, MFA, MSA and MpCA). This later activity may represent a compound activity of replacement type A and B enzymes; alternatively the new activity may be the product of a single enzyme with a hitherto unrecognized ability to deal with a broad range of substrate structures. The induction of an enzyme with broad substrate activity could replace the specialized enzymes to complete the digestion of complex plant materials. The *fae-1* gene is only strongly expressed during the early stages of fermentation on SBP, and may only contribute residual activity at later stages. The *fae-1* gene is weakly expressed throughout fermentation on WB. Therefore the FAE type B enzyme substrate utilization observed in the later stages of growth on SBP and WB may arise due to the expression of an alternative gene or genes. A BLAST-X search performed on the *N. crassa* genomic database (<http://www-genome.wi.mit.edu/>) using *Fae-1* and FAE sequences available from the NCBI database revealed five further ORFs exhibiting significant protein sequence identity. Two ORFs encode putative type A FAEs, and three encode putative type B enzymes related to the *fae-1* product reported here. The presence of these genes is consistent with the idea that *N. crassa* is able to produce more than one type of FAE, and our activity measurements, as well as the time-course mRNA expression analysis, would suggest that these act as either specialist or generalist enzymes, depending on the substrate and its degree of depolymerization. Thus *A. niger* is not the only fungus able to produce more than one type of FAE, and it is likely that most fungi will utilize a battery of such enzymes.

The *Fae-1* sequence contains two potential motifs that accord with the consensus Gly-Xaa-Ser-Xaa-Gly, which is highly conserved in serine esterases [47]. Based on the conservation of the sequence environment around these putative active-site serine residues with the *Pe. funiculosum* and the *A. awamori* esterase sequences, we predict the active site of the enzyme to be centred on serine-118 (Gly-Thr-Ser-Ser-Gly).

The *fae-1* cDNA, containing the native signal sequence, was cloned and expressed in *Pichia pastoris* using the pPIC3.5K expression vector. A representative transformant producing a high level of secreted recombinant protein was selected for production of recombinant *Fae-1* and used in large-scale culture for *Fae-1* purification and characterization. The yield of secretion reached up to 210 mg · l⁻¹ in buffered complex medium, and the recombinant protein was recovered as a single peak following hydrophobic interaction chromatography. A clear advantage of

the use of the *P. pastoris* expression system is the high yield of secreted recombinant protein against a background of very limited host protein secretion, which allows for rapid purification of the target protein [32].

The substrate recognition of recombinant *Fae-1* was characterized. As expected for a type B FAE, *Fae-1* shows high catalytic efficiency for the hydrolysis of MCA and MpCA, but no detectable activity against MSA. The catalytic efficiency of *N. crassa* *Fae-1* for the hydrolysis of Ara₂F was low (12.87 mM⁻¹ · s⁻¹) compared with that of *Pe. funiculosum* FAEB (530 mM⁻¹ · s⁻¹) [19], but was 112-fold greater than that of *A. niger* cinnamic acid esterase (CinnAE) [42] and *Pe. equi* cinnamoyl ester hydrolase (CEH or EstA') [16]. Low turnover of Ara₂F may reflect the need for longer feruloylated oligosaccharides to ensure optimal catalytic activity. The acetyl esterase activity of *Fae-1* was investigated. Even though *Fae-1* was defined as a FAE because of its ability to efficiently hydrolyse hydroxycinnamic esters, the enzyme is also able to hydrolyse acetylated substrates, but at a significantly lower efficiency. The ability of *Fae-1* to hydrolyse α -naphthyl acetate appears to be the highest activity measured for a FAE on this substrate (3 mM⁻¹ · s⁻¹), although the catalytic efficiency is 30–100-fold lower than that recorded for a true acetyl xylan esterase [50–54].

Kinetic studies performed with recombinant *Fae-1* and the synthetic monomeric substrates MCA, MFA and MpCA revealed substrate inhibition at higher substrate concentrations. These data were related to the following equation with respect to substrate inhibition: $v = (V_{\max}[S]) / (K_m + [S] + ([S]^2/K_i))$. It is therefore possible for a second substrate molecule to bind to the enzyme–substrate complex ES to produce an inactive complex, SES. The mechanism would be analogous to that usually considered for uncompetitive inhibition with a non-productive substrate-binding site in addition to the productive binding site. Certain fungal FAEs have been characterized as modular enzymes containing a catalytic domain linked to a non-catalytic domain, such as a specific carbohydrate-binding module [30]. Modular esterases from *Pe. funiculosum* [19], *Pir. equi* [16] and *Trichoderma reesei* [55] have been shown to contain a non-catalytic CBD. CBDs act to bring the catalytic site spatially closer to its substrate and to create a microenvironment where substrate is easily accessible. An enzyme subject to substrate inhibition cannot bear such a binding domain without compromising its catalytic activity, even when the substrate may be limiting in the wider environment. In the light of this information, it is perhaps not surprising that the *Fae-1* sequence does not feature a recognizable CBD. In the absence of a CBD, concentration-dependent substrate inhibition could be utilized by micro-organisms to regulate substrate conversion. The enzyme activity would be attenuated in the presence of high environmental substrate concentrations, in order to prevent the accumulation of toxic products that could not be metabolized immediately. Indeed, phenolic acids are used to inhibit microbial growth. We may speculate that the *N. crassa* enzyme has evolved to feature concentration-dependent substrate inhibition in order to regulate the activity of a secreted esterase working remote from the organism. In contrast, concentration-dependent substrate inhibition would be an ineffective way for micro-organisms to regulate the activity of enzymes carrying CBDs, because of the very intimacy of the contact between the enzyme and its potential substrate. The *N. crassa* genome also contains an ORF encoding a modular enzyme which may be identified as a putative FAE with a fungal-type CBD (45 % identity with *Fae-1* from *N. crassa*; 44 % identity with FAEB from *Pe. funiculosum*). *N. crassa* therefore has the genetic capacity to produce more than one type B FAE and, as a consequence of their modular structure,

the organism can tailor its response to substrate availability in the environment. In the present study, substrate inhibition has been detected with synthetic substrates that may be structurally similar to the products released by the action of other host enzymes. However, it would be of interest to know if higher-order structures released from plant cell walls will produce substrate inhibition, in which case the organism may have evolved its enzymes not only to avoid toxicity, but also to regulate the rate at which a complex substrate is dismantled. In microbial communities, the ability of an organism to tolerate the actions of its neighbours while maintaining its own nutritional requirement will determine species succession in the environment. It is notable that in this respect *N. crassa* is an opportunist, being the first to colonize a nutrient source and complete its life cycle before giving way to other species.

In conclusion, we have demonstrated the ability of the filamentous fungus *N. crassa* to produce multiple FAE activities. We have also shown that the expression of these enzymes is regulated in response to the initial substrate and the degree of substrate degradation as a function of time. Moreover, we have demonstrated transcriptional regulation of a gene encoding a non-modular FAE, and that the purified product of this gene is subject to substrate inhibition, which has not been reported previously for any FAE. A search of the *N. crassa* genome sequence has revealed three further ORFs with protein sequences similar to Fae-1, including a putative enzyme carrying a CBD. We suggest that FAEs have evolved to operate over a wide range of substrate availability, but at the same time have an innate ability to modulate their catalytic activity in respect of the catabolic requirements and the product sensitivities of the organism that secretes them.

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